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PHYSIOLOGY AND BIOCHEMISTRY OF THE  
FACULTATIVELY ANAEROBIC CHEMOLITHOTROPH  
THIOBACILLUS DENTRIFICANS GROWN IN  
CHEMOSTAT CULTURE

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A thesis submitted in fulfilment of the requirements for the  
degree of Doctor of Philosophy

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ABBREVIATIONS

ADP =	Adenosine 5'diphosphate
AMP =	Adenosine 5'monophosphate
APS =	Adenosine phosphosulphate
ATP =	Adenosine 5'-triphosphate
NAD <sup>+</sup> =	Nicotinamide adenine dinucleotide
NADH =	Reduced nicotinamide adenine dinucleotide
DNP =	2, 4-Dinitrophenol
[H] =	Hydrogen equivalents
D =	Dilution rate
$\mu$ =	Specific growth rate
m =	Maintenance coefficient
D crit =	Critical dilution rate
Y <sub>g</sub> =	True growth yield
Y <sub>ATP</sub> =	Molar growth yield per mole ATP
qS <sub>2</sub> O <sub>3</sub> =	Specific rate of thiosulphate consumption
m <sub>ATP</sub> =	Maintenance coefficient per mole ATP
1/Y =	Reciprocal of yield
1/D =	Reciprocal of dilution rate

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### SUMMARY

A continuous culture of *T. denitrificans* was established in media which had been previously found suitable in batch cultures. The response of the organism to dilution rate was studied in relation to its maximum growth rate, and possible change in the pattern of products from thio-sulphate and nitrate as substrates. The organism was grown aerobically and anaerobically with the limitation of thiosulphate, tetrathionate or nitrate, and the effect of this on the D crit. and the patterns of substrate use, growth yield and maintenance energy determined. The comparison of growth yield and substrate conversion efficiencies between aerobic and anaerobic conditions provided a unique opportunity to compare a respiratory system in which electrons pass from substrate directly to cytochrome c then to oxygen or nitrate. Anaerobically the Y<sub>g</sub> was 70% of that aerobically. Prolonged aerobic cultures retained their capacity to grow anaerobically. The use of the chemostat with a limiting oxidant enabled the use of normally toxic substances such as nitrite to be used as growth substrate in the solution phase and nitrous oxide in the gas phase. Nitrate reductase was repressed by oxygen and nitrite, whereas nitrite reductase was fully derepressed by nitrate. The transition between various steady-state limiting substrates was studied to give an idea of the organism's adaptability and substrate turnover when confronted with varying conditions. The presence of a sulphur-oxidizing and a thio-sulphate-oxidizing enzyme was investigated in an attempt to elucidate part of the thiosulphate pathway in this organism.

Calculating from the energy requirement for CO<sub>2</sub>-fixation, the Y<sub>g</sub> values calculated from chemostat data indicated ATP production to be 4-5 mol/mol thiosulphate and 8-9 mol/mol tetrathionate for anaerobic cultures, and 6-7 mol/mol thiosulphate for aerobic cultures.

Inhibitor experiments with non-growing suspensions indicated oxidative phosphorylation to occur both aerobically and anaerobically.

PART 1: GENERAL INTRODUCTION

## PART 1: GENERAL INTRODUCTION

### 1.1 Historical background and physiology of Thiobacilli

The first Thiobacillus species was discovered in 1902 by Nathansohn, when he probably isolated something very similar to Thiobacillus neapolitanus, but it was not until 1904 that Beijerinck gave the species the name of Thiobacillus. This group of organism uses the oxidation of sulphur compounds to sulphate as their principal, and sometimes their only, source of energy for growth. They represent a very special type of metabolism by growing and multiplying in the absence of any organic nutrients by synthesising all their constituents from carbon dioxide and inorganic salts. Hence they are classified as 'chemolithotrophic autotrophs' (Schlegel, 1975; Whittenbury and Kelly, 1977).

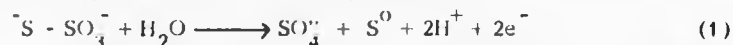
Since their discovery many studies have been carried out to elucidate the mechanisms involved in the oxidation of various sulphur compounds. However, it is still not precisely clear what these mechanisms are in individual species. This has been partially due to the lack of analytical methods. Further complications arose through chemical reactions occurring between intermediates which are not attributed to the bacteria. For example, Vishniac and Santer (1957) found that if thiosulphate is partially oxidized to tetrathionate, so that both are present simultaneously for several hours, then some tetrathionate will dismute to trithionate and pentathionate. In recent years, increased use of bacterial extracts such as cell free membranes or particulate forms, and more refined methods such as chromatography and radiochemical techniques (Skarzynski and Ostrowski, 1958; Santer, 1959; Peck and Stulberg, 1962; Suzuki, 1965b; Kelly and Syrett, 1966b) has led to more information being obtained on the energy metabolism.

The activities of Thiobacilli have been reviewed at regular intervals by Vishniac and Santer, 1957; Peck, 1962; Trudinger, 1967; Kelly, 1968; Suzuki, 1975. Much of this work has been done using thiosulphate as the experimental substrate, from which the end products have long been known to be sulphate, sulphur and tetrathionate (Nathansohn, 1902; Beijerinck, 1904; Starkey, 1935; Parker and Prisk, 1953). Thiobacilli are fully

capable of carrying out complete oxidation to sulphate under suitable conditions, but numerous factors have been found to cause a variety of end products to be produced. Vishniac and Trudinger, (1962) <sup>and</sup> Trudinger, (1964d, 1964e) found that factors such as substrate, oxygen or cell concentrations and strain of organism used could alter the relative amounts of products formed.

### 1.2 The mechanism of sulphur compound oxidation

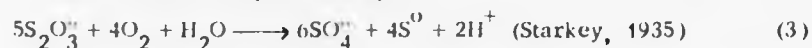
Already in the early studies of *Thiobacilli*, two divergent views arose on the initial reactions of thiosulphate oxidation. The first one suggested that thiosulphate was oxidized to sulphate and sulphur by the cleaving of the sulphur-sulphur link in the thiosulphate ion:-



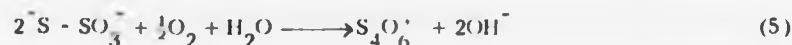
Beijerinck's (1904) 1:1 ratio of sulphur to sulphate fits this scheme:-



or even when data show more sulphate than sulphur is produced due to further oxidation of sulphur to sulphate:-



The alternative view was that the pathway to sulphate was through a sequence of polythionate intermediates and the sulphur-sulphur bond was cleaved at the later stages (Famiya, Haga and Huzisige, 1941; Vishniac and Santer, 1957). Tetrathionate production, postulated as the first step, is a simple process involving the oxidative condensation of two thio-sulphate ions:-

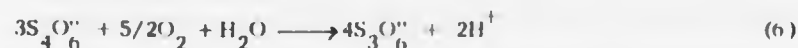


A thiosulphate oxidizing enzyme isolated by Trudinger (1961) from

*Thiobacillus neapolitanus* strain X was found to catalyse the reaction

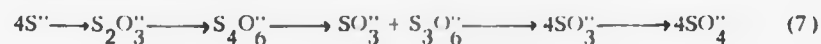


Vishniac and Santer (1957) postulated a further oxidation of tetrathionate to trithionate:-



Trudinger (1964b,c) found that trithionate metabolism could be sparked off in the presence of thiosulphate and would continue rapidly by hydrolytic scission in dense cell suspensions at low oxygen tensions to thiosulphate and sulphate. The transient occurrence of one or both polythionates has been demonstrated by numerous workers (Nathansohn, 1902; Santer *et al.*, 1960; Trudinger, 1959, 1961, 1967; Jones and Happold, 1961; Okuzumi and Kita, 1965; Kelly and Syrett, 1966b). Starkey (1934a, b) and Vishniac (1952) suggested the possible intermediate of dithionate, but this was later regarded as unlikely (Vishniac and Santer, 1957) although Lieske (1912) obtained good growth of *Thiobacillus denitrificans* on dithionate and Cook (1967) showed that a nucleotide-bound dithionate may be formed by *Thiobacillus denitrificans*. Other workers have postulated the involvement of organic sulphur compounds as intermediates (Skarzynski and Ostrowski, 1958), perhaps in the conversion of trithionate to sulphate (Jones and Happold, 1961).

There is also evidence of sulphite being the last intermediate before sulphate formation (Deley and Van Poucke, 1961; Trudinger, 1964d). Further studies by London and Rittenburg (1964) on sulphide, thiosulphate, tetrathionate and trithionate showed that all these were oxidized to sulphate, with sulphide and thiosulphate showing accumulations of tetrathionate and trithionate. It was therefore postulated that a reaction sequence took place as follows:-

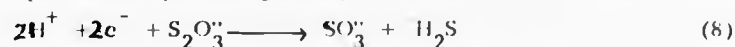


Attempts were made to block sections of this pathway by thiol-binding reagents such as N-ethylmaleimide (Trudinger, 1965) to show that intermediates such as tetrathionate were on the pathway to sulphate. This was not conclusive as tetrathionate could also be produced by the malfunctioning of a poisoned enzyme. Similar attempts were carried out by Sinha and Walden (1966).

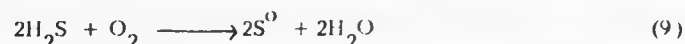
Since these early views on the pathway of sulphur oxidation, other possibilities have arisen. Peck (1960) described cell free preparations of *T. thioparus* producing sulphate and sulphur without any polythionates



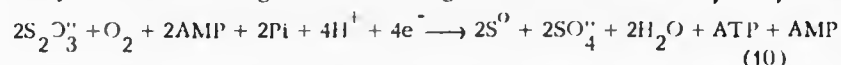
as intermediates. In this case the initial reaction was the reductive cleavage of thiosulphate to give sulphide and sulphite.



Sulphur was produced from the sulphide due to the presence of sulphide oxidase in the extract



The sulphite ions were converted to sulphate through a process involving a nucleotide-bound sulphate, adenosine phosphosulphate (APS) by enzyme-catalysed reactions to give the following overall reaction (Peck, 1960, 1962):-



This gives the 1:1 ratio of sulphur:sulphate as suggested by Beijerinck (1904). Peck (1962) also showed that the oxidation of sulphide to sulphate was coupled with phosphate esterification.

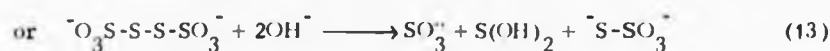


The thiosulphate then follows the same pathway involving APS.

The production of sulphur or tetrathionate by sulphide or thiosulphate oxidation would be a side reaction and not concerned with the main oxidative pathway. In this scheme, Peck and Stulberg (1962) showed that the sulphite received a fourth oxygen atom from phosphate, in the reaction with adenosine monophosphate (AMP) and phosphate, to form sulphate. By using labelled  $^{18}\text{O}$ , Santer (1959) found that 22-23% of the sulphate produced was labelled with the isotope. Since APS reductase comprises 3-4% of the cell protein in *T. thioparus* (Lyric and Suzuki, 1970) and 4-5% in *T. denitrificans* (Bowen, Happold and Taylor, 1966) the APS pathway must play an important role in the energy metabolism of these organisms.

There are several arguments against the polythionate theory. First, no account is given of the observed quantitative sulphur precipitation by many *Thiobacilli* other than it arises by chemical interaction among intermediate polythionates. Second, little is known of the enzymatic reactions for polythionate oxidations. It is not certain whether they are true intermediates or side products of unstable enzyme-bound inter-

mediates. Third, Trudinger (1964d, e) found that tetrathionate and trithionate oxidations appeared to require low oxygen concentrations and occurred best in anaerobic conditions in the early stages before sparking of the reaction by thiosulphate. Fourth, there is a reasonable amount of evidence suggesting the initial steps in metabolism of polythionates were to produce short chain sulphur-compounds and not direct intermediates in thiosulphate oxidation. For example, Thiobacillus neapolitanus X catalyses the hydrolysis of tetrathionate as follows (Trudinger, 1964a, 1967):-



Similarly, trithionate can be dismutated hydrolytically (Iluzisig and Haga, 1944; Trudinger, 1964b, 1967):-

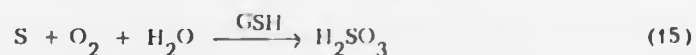


Other examples include work by Imai, Okuzumi and Katagiri (1962), Okuzumi (1965, 1966). Fifth, studies by Starkey (1934b) showed that no polythionates accumulated when Thiobacillus thiooxidans, T. thioparus and Thiobacillus novellus were grown on thiosulphate. He suspected that the presence of any incompletely oxidized sulphur materials was a result of the activity of certain heterotrophic microorganisms that have this capacity. Sixth, through the use of  $^{35}\text{S}$ , Kelly and Syrett, 1966b; Kelly, 1968, give evidence which is consistent with the oxidation mechanism based on Peck's hypothesis (1962), but also is consistent with a modified polythionate oxidation pathway. By using  $^{35}\text{S}$  labelled thiosulphate in both sulphur atoms, the  $^{35}\text{S}$  trithionate produced was found to give two rates. This indicated the initial cleavage of thiosulphate to sulphide and sulphite in which the sulphite then reacts with thiosulphate to produce trithionate. However, the sulphide must first form sulphite before proceeding, thus producing a slower reaction. The trithionate production is shown as a side reaction as sulphate is produced as a result of the oxidation of sulphite. Further evidence for Peck's theory was apparently demonstrated by Ostrowski and Krawczyk (1957). The sulphate produced from the sulphite remained in solution while the sulphide entered the cell and was

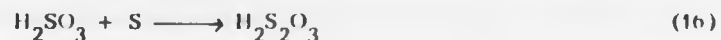
released in part as sulphur and part as sulphate. Peck and Fisher (1962) also demonstrated the preferential incorporation of the outer sulphur-atom into sulphur, by adding excess elementary sulphur to T. thioparus oxidizing  $^{35}\text{S}$  labelled thiosulphate, where the sulphite is simultaneously converted to sulphate.

Further studies of Thiobacilli have been carried out on elementary sulphur. It was found by Vogler and Umbreit (1941) that the physical form of sulphur influences its oxidation with the rate increasing with decreasing particle size. Vishniac and Santer (1957) suggested the need for substrate amounts of reduced glutathione to catalyse the oxidation of sulphur. Suzuki and Werkmann (1959) also indicated this and presumed it to be oxidizing sulphide from sulphur reduction. Sulphur oxidation has also been thought to initially involve either its reduction to sulphide or for there to be an intermediate stage in the oxidation of sulphide (Parker and Prisk, 1953; Suzuki and Werkmann, 1959; Peck, 1962; Trudinger, 1967). The mechanism of sulphur uptake was intensively studied and summarised by Roy and Trudinger (1970). Studies using  $^{32}\text{S}$  (Kaplan and Rittenburg, 1962) showed that the sulphate produced from sulphide was very rich in  $^{32}\text{S}$ , whereas the sulphate from sulphur was not. From this it was presumed that the isotope fractionation occurred at the cell boundary and that sulphur was transferred across the membrane without prior reduction to sulphide.

In 1965 Suzuki and later Suzuki and Silver (1966) partially purified an enzyme that catalyses sulphur oxidation in the presence of oxygen with only catalytic amounts of reduced glutathione. From T. thioparus the first detectable product was found to be sulphite:-



Thiosulphate was produced by a non-enzymatic condensation of sulphur and sulphite:-



Suzuki (1965a) suggested that sulphur oxidation may initially involve the  $\text{S}_8$  ring being opened by the nucleophilic attack of reduced glutathione to

produce a polysulphide. This may then be enzymatically oxidized by both oxygenase and oxidase reactions to release successive sulphite ions:-



As only sulphite has been found during these reactions, it could mean that no free intermediates are produced due to the reaction occurring on a single enzyme protein. The oxidation of sulphite could be by the AMP-dependent mechanism (Peck, 1962) or independent of AMP by the oxidase shown in *T. novellus* (Charles and Suzuki, 1965, 1966) and *Thiobacillus concretivorus* (Moriarty and Nicholas, 1970a). Moriarty and Nicholas (1969, 1970a, b) postulated sulphide oxidation proceeded in two stages in *T. concretivorus*. The first stage involved sulphide losing 2 electrons, leading to polymerisation of the resulting sulphur atoms. The free sulphide present until the end of this stage may react with the developing polymeric sulphur chains to form polysulphides. The second stage would be the subsequent oxidation of short chain polysulphides to longer membrane bound polymeric sulphur compounds, with subsequent oxidation by a mechanism similar to Suzuki (1965) and Suzuki and Silver (1966).

Taylor (1968) found that a *T. neapolitanus* extract was capable of oxidizing sulphur to sulphate in the absence of reduced glutathione. Thiol binding reagents inhibited sulphur oxidation, indicating that a sulph-hydryl group was involved in the cleavage of the sulphur molecule, though no clear explanation could be made to account for this reaction at the time. Trudinger (1964a) claimed the formation of sulphur from thiosulphate was governed by experimental conditions. Later, Kelly and Syrett (1966) found no  $^{35}S$  in sulphur during simultaneous oxidation of sulphur and thiosulphate ( $^{35}S^- - SO_3^-$ ).

The initial step of thiosulphate oxidation is probably its cleavage to sulphite and sulphane<sup>-sulphur</sup> moieties (Kelly, 1968; Suzuki, 1975) for which rhodanese has been suggested as catalyst (Tabita, Silver and Lundgren, 1969; Sörbo, 1975; Suzuki, 1975). This enzyme has been found in many

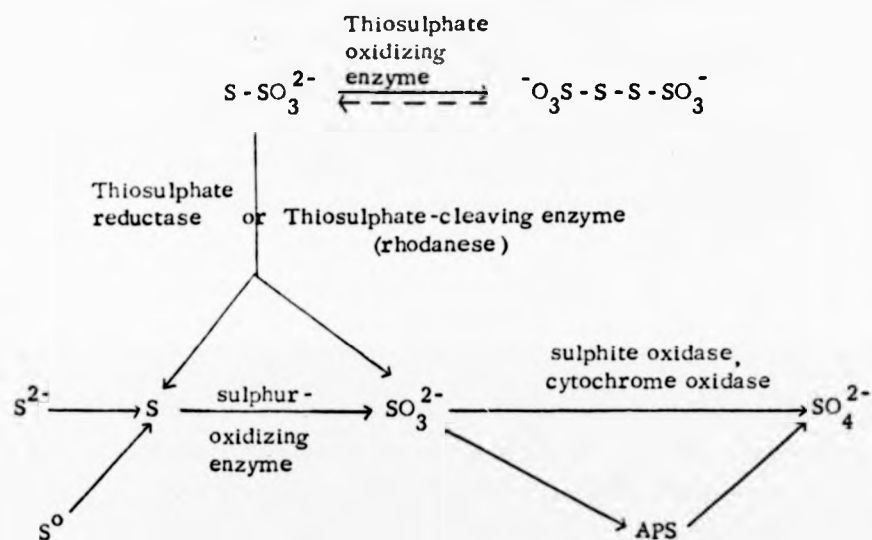
thiosulphate oxidizing bacteria (McChesney, 1958; Bowen, Butler and Happold, 1965; Charles and Suzuki, 1966a; Yoch and Lindstrom, 1971; Guay and Silver, 1975; Kelly and Tuovinen, 1975; Sörlö, 1975). The enzyme will act with various sulphur donors and acceptor compounds (Westley, 1973), including cyanide acting as an acceptor for the sulphane-sulphur atom which has been found to increase the bacterial yield in T. denitrificans (Bowen, Butler and Happold, 1965).

To summarise, most of the experimental results in the past can be explained by Scheme 1 (Suzuki, 1975) where all the enzymes involved have been isolated and characterised with the exception of tetrathionate reductase which may be similar to thiosulphate reductase or rhodanese. When sulphide or sulphur is the substrate for oxidation, it enters the main pathway directly. Thiosulphate oxidation starts with its cleavage to sulphane-sulphur and sulphite. Sulphite is oxidized to sulphate by one of two pathways. Sulphane-sulphur is oxidized to sulphite, but when the oxidation rate is slower than the rate of thiosulphate cleavage, it is excreted outside the cells as elemental sulphur. When thiosulphate cleavage to <sup>sulphane-</sup> sulphur and sulphite is hindered, it is oxidized to tetrathionate. Little energy is obtained by this process, as only 0.5 mole oxygen is required per mole thiosulphate, hence only 0.5 mole ATP is produced, which thermodynamically is only about 5% of the total energy. Tetrathionate accumulated is possibly oxidized further after a hydrolytic cleavage to thiosulphate, sulphur and sulphite (Trudinger, 1964a,c). Once thiosulphate is cleaved, sulphite inhibits the thiosulphate oxidizing enzyme (Lyric and Suzuki, 1970). Trithionate will form chemically from tetrathionate and sulphite (Trudinger, 1964a) and so will accumulate when the rate of sulphite formation is faster than that of sulphite oxidation.

### 1.3 Energy metabolism in Thiobacilli

The bioenergetics and energy coupling of the sulphur metabolism of Thiobacilli have been reviewed in the past few years (Peck, 1968; Kelly, 1971; Suzuki, 1975; Kelly, 1978). In 1956 Aubert et al.

SCHEME 1: Thiosulphate oxidation (Suzuki, 1975)



demonstrated that carbon incorporation into *Thiobacilli* cells was dependent on the oxidation of thiosulphate. Other studies of that time (Santer and Vishniac, 1955; Trudinger, 1955, 1956) showed that the enzymes needed for carbon dioxide to be assimilated into the Calvin carbon cycle were present in *T. denitrificans* and *T. thioparus*. The driving forces for this carbon reduction cycle and other biosynthetic reactions are adenosine triphosphate (ATP) and reduced pyridine nucleotides (NADH) (Ross *et al.*, 1968). The generation of NADH by thiosulphate is an energy linked process relying upon a reversed electron flow driven by ATP (Peck, 1968). This is the main energy need that chemolithotrophs have to supply, and accounts for at least 80% of their total energy requirement for cell biosynthesis (Forrest and Walker, 1971).

$6\text{CO}_2 + 18\text{ATP} + 12\text{NADH}_2 \longrightarrow \text{Fructose-6-Phosphate} + 12\text{NAD} + 17\text{Pi} + 18\text{ADP}$  (19)

Peck (1960) showed one possible mechanism by which the necessary energy could be obtained. This is by substrate-level phosphorylation where one ATP is produced from the oxidation of two thiosulphate molecules and is not uncoupled by 2, 4-dinitrophenol. The process involves the production of adenosine phosphosulphate (APS) from sulphite which on phosphorylation produces ATP. This mechanism has not been found in all *Thiobacilli* due to the lack of evidence for the enzymes.



Later, Hempfling (1964) found that *T. neapolitanus* produced more ATP than could be accounted for by substrate level phosphorylation alone.

Evidence for the presence of various cytochromes of the c and a type has been reported in several *Thiobacilli* (Szczepkowski and Skarzynski, 1953; Trudinger, 1961; Ross *et al.*, 1968) and that the electron transport from thiosulphate to molecular oxygen or nitrate is coupled to ATP formation, *i.e.* oxidative phosphorylation. The production of ATP during the oxidation of sulphite to sulphate by the APS pathway, and sulphite oxidase with cytochrome c as electron acceptor has now been established (Peck, 1960). *T. thioparus* has both APS reductase and sulphite oxidase, which poses an interesting question about their respective roles in this organism (Lyric and Suzuki, 1970). Whereas *T. novellus* (Charles and Suzuki, 1965) and *Thiobacillus intermedius* (Charles, 1969) only have sulphite oxidase present. The advantage of the APS system is that if the electrons produced are directed through a cytochrome system, a further yield of ATP would arise from ADP by oxidative phosphorylation.

#### 1.4 *Thiobacillus denitrificans*

The *Thiobacillus* genus contains a variety of species varying considerably in physiological characteristics (Vishniac and Santer, 1957; Trudinger, 1967; Hutchinson *et al.*, 1969), so inevitably there has been much discussion and comparison of the oxidation of various sulphur compounds in an attempt to bring them together as one theory. All the species oxidize thiosulphate and sulphide using molecular oxygen except for one species, *T. denitrificans*, that can grow anaerobically on sulphur or thiosulphate whence it reduces nitrate to nitrogen instead of reducing oxygen (Baalsrud <sup>Taylor *et al.*, 1971;</sup> and Baalsrud, 1954; Taylor and Hoare, 1971; Timmer-ten Hoor, 1976). Two other species, *T. thioparus* and *Thiobacillus thiooxydans*, can also grow anaerobically on thiosulphate with nitrate as an electron acceptor, but this is only reduced to nitrite, not to nitrogen (Skarzynski and Ostrowski (1958).

*T. denitrificans* was first isolated by Beijerinck in 1904, and the first study on it was carried out by Lieske in 1912. It was not until 1954 that an extensive study of its physiology was carried out by Baalsrud and Baalsrud. Since then, this strictly chemoautotrophic bacterium has

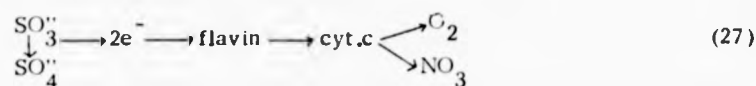
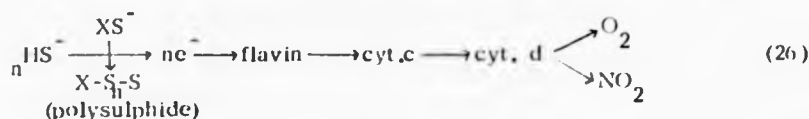
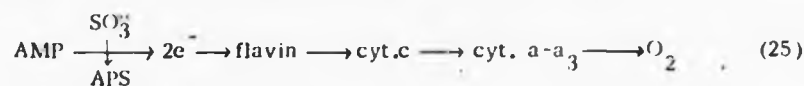


undergone extensive enzymological studies (Aubert *et al.*, 1959; Bowen *et al.*, 1966; Sargeant *et al.*, 1966; Peeters and Aleem, 1970a, b; Adams *et al.*, 1971a, b; Schedel *et al.*, 1975; Sawhney and Nicholas, 1977, 1978a, b) until in 1976 Timmer-ten Hoor investigated the energetic aspects of its metabolism of reduced sulphur compounds.

The intact cells have been shown to be capable of oxidizing thio-sulphate, sulphide and sulphite with either nitrate or molecular oxygen as the terminal electron acceptor (Aleem, 1975). This involves the participation of the electron transport chain (Peeters and Aleem, 1970b). The whole process provided electrons and hydrogen equivalents to produce ATP and NADH for assimilation of carbon dioxide to cell carbon.

The cleavage of thiosulphate to sulphide and sulphite moieties is followed by their further oxidation to sulphate involving the transfer of electrons to nitrate if anaerobic or oxygen if aerobic. Aminuddin and Nicholas (1974b) (Milhaud *et al.*, 1958; Peeters and Aleem, 1970b; Adams *et al.*, 1971a, b) found that these moieties act as reductants for nitrate and nitrite reductase which had earlier been isolated by Adams *et al.*, 1971b; Aminuddin and Nicholas, 1973; Baldensperger and Garcia, 1974. Two systems were found by using different extracts. In the supernatant fraction sulphite was converted to APS in the presence of AMP, releasing two electrons that were passed along an electron transport system mediated by flavin, cytochrome c, cytochrome a-a<sub>3</sub> and, finally, oxygen. Adenosine-5'-phosphosulphate reductase (Bowen *et al.*, 1966; Sargeant *et al.*, 1966; Aminuddin and Nicholas, 1974a) consisted of 4-5% of the protein and was therefore thought to play a major role in thiosulphate metabolism. Adenylate kinase (Sargeant *et al.*, 1966) enables the ATP to be produced from APS. In the membrane fraction, the sulphide moiety reacted with a sulphur-protein acceptor to form polysulphides, releasing electrons which passed via flavin, cytochrome c, cytochrome d and oxygen or nitrite. The electrons produced when sulphite was oxidized to sulphate were passed to flavin, cytochrome c and oxygen or nitrate. With the use of inhibitors, Aminuddin and Nicholas (1974b) proposed that sulphite was linked to nitrate reductase, and sulphide to nitrite reductase. Adams *et al.* (1971b)

suggested that the sulphite dependent nitrate reductase catalysed the reduction of nitrate by a Ping Pong mechanism:-



Sawhney and Nicholas (1977) found that NADH could also be an effective electron donor for nitrate reductase. The presence of cytochromes in *T. denitrificans* has been extensively investigated (Aubert *et al.*, 1959; Peeters and Aleem, 1970b; Ishaque and Aleem, 1971), as have the cytochrome oxidase components (Aubert *et al.*, 1959).

Other enzymes involved in the metabolism of reduced sulphur compounds have been found. These include (i) rhodanese by Bowen *et al.*, 1965; and Sargeant *et al.*, 1966, which is involved in the cyanide dependent cleavage of thiosulphate, (ii) sulphite oxidase by Aminuddin and Nicholas (1974a) involved in the electron transport chain, (iii) sulphite reductase by Schedel *et al.*, 1975, who believe it to have a dissimilatory role in the overall oxidation of reduced sulphur compounds.

The assimilation of carbon dioxide into the Calvin carbon cycle of *T. denitrificans* was first demonstrated by Trudinger (1955, 1956). Since then, Peeters and Aleem (1970a) and Taylor and Hoare (1971) have found that the enzymes involved in the tricarboxylic acid cycle (TCA) were all present except for  $\alpha$ -ketoglutarate dehydrogenase and succinyl CoA synthetase. Under autotrophic conditions, the Krebs cycle seems to lose its energetic functions and only fulfils the organism's biosynthetic needs for which  $\alpha$ -ketoglutarate dehydrogenase is not necessary (Smith *et al.*, 1967), though the absence of this enzyme is not considered a causative feature of obligate autotrophy. It has been found to be absent in *Thiobacillus A2* when

grown autotrophically, but present when grown heterotrophically (Peeters and Aleem, 1970a). The glyoxylate pathway was also found, but it is not known if it is more important than the TCA cycle (Peeters and Aleem, 1970a).

Much work has been carried out to find the intermediates involved in nitrate reduction (Aminuddin and Nicholas, 1973; Ishaque and Aleem, 1973; Aleem, 1975; Baldensperger and Garcia, 1975). This has given rise to the proposed pathway mediated by nitrate, nitrite, nitric oxide, nitrous oxide and nitrogen.

Most of the studies on T. denitrificans have been carried out in batch cultures with the exception of those by Timmer-ten Hoor (1976) who used continuous flow culture at one <sup>dilution</sup> rate. To study the behaviour of a microorganism it is best for it to be growing in conditions where all is constant except for the one condition being studied. This cannot be achieved in a batch culture which is a discontinuous process where the environment and the organisms are constantly changing. The continuous flow culture thus enables a steady state to be maintained, theoretically indefinitely, where the organism can be studied, giving reliable and reproducible data (Bull, 1974). The study of T. denitrificans by standard procedures in continuous chemostat culture would enable the estimation of true growth yields, which generally increase as the growth rate increases, and maintenance coefficients (Pirt, 1965), for comparison with other Thiobacilli. Bauchop and Elsden (1960) proposed that the amount of growth of an organism was directly proportional to the amount of ATP that could be obtained from the degradation of the energy source in the medium (Stouthamer, 1977). Two kinds of calculation can be derived using the data, which enables an assessment of the biochemical energy (ATP and NADH) generated during thiosulphate oxidation, (a) energy coupling calculated from growth linked carbon dioxide fixation, (b) based on carbon assimilation data (Kelly, 1978). As the molar growth yield of ATP is influenced by its growth conditions, i.e. types of media, then the chemostat means of analysis would enable accurate results in specific conditions to be obtained.

#### 1.5 The aims of the present project

The lack of information of *Thiobacillus denitrificans* grown under continuous chemostat conditions led to the present study which is outlined below:

1. Establishment of continuous culture.
2. Determination of  $\mu_{max}$ ,  $D_{crit}$ ,  $Y_g$ ,  $m$  values.
3. Effect of dilution rate on yield.
4. Effect of oxygen on yield.
5. Range of sulphur compounds which it is capable of oxidizing.
6. Range of nitrogenous compounds as alternative electron acceptors to oxygen.
7. Calculation from data of energetic properties and characteristics compared with other organisms.
8. Enzymological study of some of the enzymes involved in nitrogenous compound reduction.
9. Check on the role of an 'oxygenase' in a facultative anaerobe.
10. Distribution of energy conservation sites.

PART 2: MATERIALS AND METHODS

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### 2.1 Organism

Thiobacillus denitrificans strains newly isolated from enrichment cultures, and a culture collection strain NCIB 9548 were maintained anaerobically in completely filled 100 ml bottles at 30<sup>0</sup> C, or aerobically in flasks shaken at 30<sup>0</sup> C. These were subcultured once every seven days. A further stock was maintained on agar slopes in universal bottles fitted with a suba-seal, flushed with oxygen free nitrogen. These were subcultured every month and once grown maintained at 4<sup>0</sup> C.

#### 2.1.1 Enrichment Cultures

Six soil samples and two water samples were suspended in medium of the same composition as in section 2.2.1 in 100 ml anaerobic screw topped bottles. The samples included:

- (a) top soil of flower beds
- (b) soil 5 cm below surface of flower bed
- (c) mud from fresh water stream
- (d) surface mud from sea shore
- (e) surface mud from sea shore near sewage outlet
- (f) mud 2.5cm below surface of sea shore
- (g) water from fresh water stream
- (h) water on surface of mud from sea shore.

This resulted in samples c, d, g and h giving off gas and producing a white deposit, with c and g being the most active samples. After four subcultures, agar plates of the same composition as the medium were spread with diluted samples and kept anaerobically at 30<sup>0</sup> C. Colonies of the Thiobacilli type were isolated and further subcultured until a pure culture was established. Microscopic examination showed the bacteria to be gram negative, motile and rod shaped. A strain of T. denitrificans supplied by J. Bildensperger did not display such active growth as the isolated cultures.

## 2.2 Culture conditions

### 2.2.1 Batch culture media

The medium for the bottles and flasks contained ( $\text{gl}^{-1}$  in distilled water):  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ , 5;  $\text{KNO}_3$ , 2;  $\text{NH}_4\text{Cl}$ , 1;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.4;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 1 ml of 2% (w/v) solution in 1M HCl; trace metal solution (Tuovinen and Kelly, 1973), 1 ml. This was sterilized at 15 lbs  $\text{in}^{-2}$  for 15 minutes. A  $\text{NaHCO}_3$  solution containing  $2 \text{ gl}^{-1}$  was added separately after filtering through a sterile 2 inch dia. bacterial asbestos filter sheet (Carlson-ford, grade HP/EKS) with 5 ml  $\text{l}^{-1}$  0.5 M NaOH to adjust the medium to pH 7.0.

The trace metal solution was made up of 50g EDTA (disodium salt) in 500 ml water, into which 9 g NaOH was dissolved. In this solution the following metals were added ( $\text{gl}^{-1}$ ):  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 11;  $\text{CaCl}_2$ , 5;  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 2.5;  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.5; ammonium molybdate, 0.5;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.2. The pH was adjusted to pH 6.0 with 1 M NaOH and made up to 1 litre with distilled water.

### 2.2.2 Chemostat culture media

Anaerobic nitrate-limited cultures and aerobic thiosulphate-limited cultures were grown on medium of the same composition as already mentioned, but in the absence of  $\text{NaHCO}_3$ . For anaerobic growth in nitrite-limited medium,  $\text{KNO}_3$  was omitted from the medium and a solution of  $\text{NaNO}_2$  ( $2.56 \text{ gl}^{-1}$ ) was pumped into the chemostat apparatus from a separate reservoir to avoid a chemical reaction in the bulk medium between nitrite and thiosulphate. Similarly, to obtain cultures growing on tetrathionate, a thiosulphate-free basal medium fed at pH 9.2 was employed together with a separate supply of  $\text{K}_2\text{S}_4\text{O}_6$  solution ( $6.06 \text{ gl}^{-1}$ ) at pH 3.5, the two mixing in the chemostat vessel and being automatically maintained at pH 7.0. For anaerobic thiosulphate limitation,  $\text{KNO}_3$  was supplied as  $3 \text{ gl}^{-1}$ . The anaerobic thiosulphate-limited ( $5 \text{ gl}^{-1}$ ) culture grown on nitrous oxide was at first maintained with a mixture of nitrate ( $3 \text{ gl}^{-1}$ ) and nitrous oxide. Later it was grown on nitrous oxide only;  $20 \text{ ml N}_2\text{O min}^{-1}$  was mixed with  $100 \text{ ml } 5\% \text{ CO}_2$  in  $\text{N}_2 \text{ min}^{-1}$  and passed through the culture.

### 2.2.3 Chemostat construction

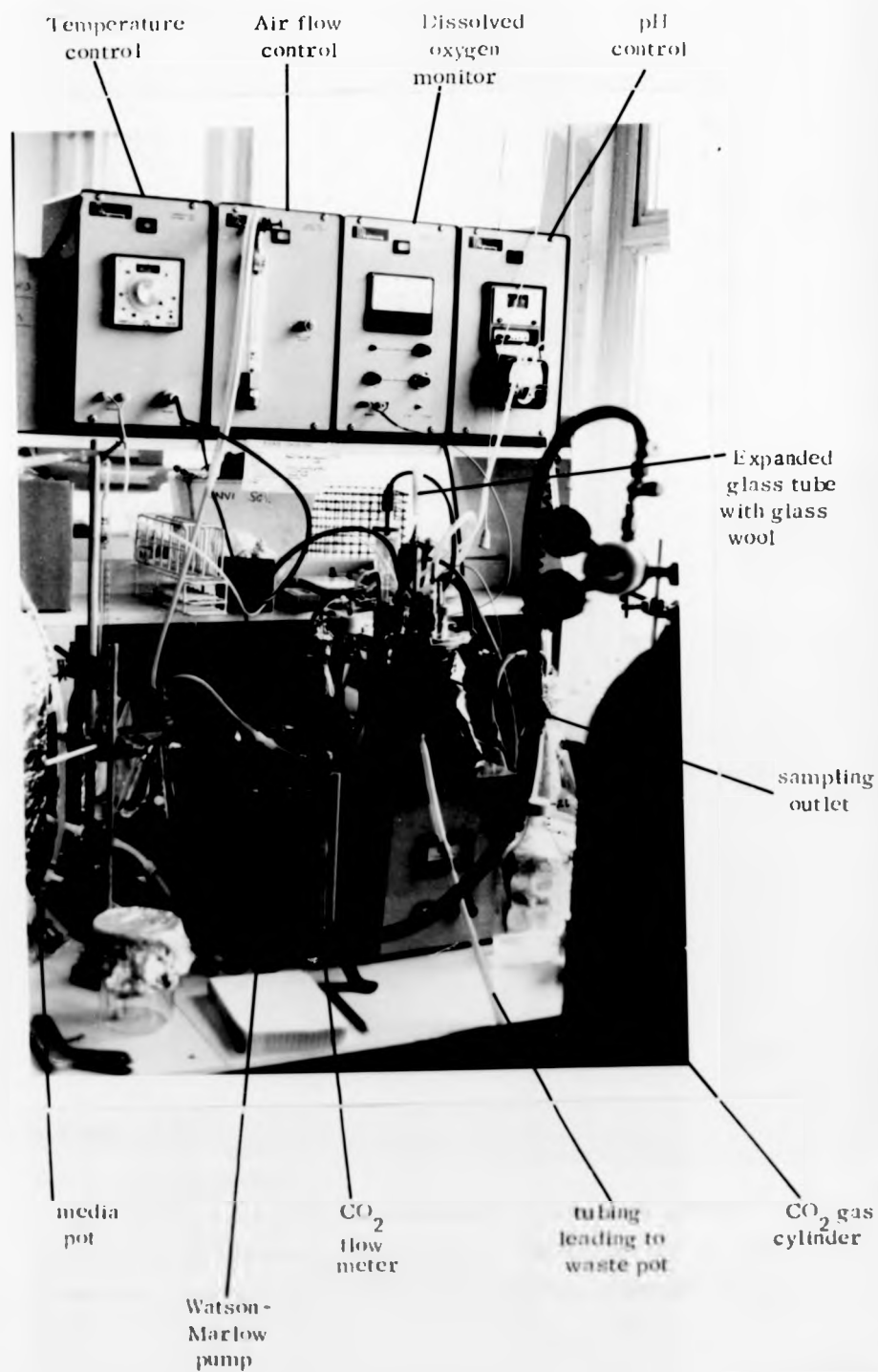
Two types of chemostat were used. Most of the studies were carried out in an LH modular type series 500 fermenter (LH Engineering, Slough, Buckinghamshire), Fig. 1. This consisted of a culture volume of 750 ml in a 1 l vessel, provided with agitation ( $750 \text{ rev min}^{-1}$ ), temperature control ( $30^\circ \text{C}$ ), pH control by the automatic addition of 0.5 M or 2 M NaOH to maintain culture at pH 7.0, and aeration. The inflow of all gases was measured and controlled by a flow meter with a fine needle valve and sterile filters. Two types of filters were used: (1) expanded glass tubes filled with glass wool, (2) in line air filters from Microflow Ltd. (Hampshire). Medium solutions were pumped into the vessel through silicone tubing via two types of filters by means of Watson Marlow MHRE 7 flow inducers (Watson Marlow, Falmouth, Cornwall). The filters used were (1) in line filter unit (Whatman Gamma 12) with glass fibre filter, (2) expanded glass tubes filled with glass wool. The gases were passed through black butyl rubber tubing in both aerobic and anaerobic conditions. All tubing was attached by means of 'shuco' clips and hose clips. Possible wall growth in the culture vessels was minimised by coating them with dichlorosilane applied as 5% solution in chloroform.

Anaerobic cultures were continuously flushed with 5% (v/v)  $\text{CO}_2$  in nitrogen at  $15 \text{ ml min}^{-1}$  for a 750 ml culture. The effluent gas from the culture was passed through a Drechsel bottle trap containing pyrogallol. The medium reservoir vessels (20 l glass aspirators) were held under nitrogen to maintain the anaerobic conditions. This was carried out by attaching a rubber football or rugby bladder containing nitrogen to the vessel. This was regularly refilled. Before the chemostat was inoculated, the whole system including the waste medium vessel (20 l glass aspirators) were flushed with oxygen free nitrogen.

Aerobic cultures were flushed with air at  $150 \text{ ml min}^{-1}$  at first, and later with the addition of supplementary carbon dioxide at  $7 \text{ ml min}^{-1}$ . The amount of dissolved oxygen in the culture was monitored by a dissolved oxygen unit from LH Engineering with a steam sterilizable oxygen electrode.



FIG. 1 LH MODULAR TYPE SERIES 500 FERMENTOR



The effluent gas from the medium and waste vessels and chemostat were allowed into the atmosphere. Some aerobic cultures were grown in an LHCC 1500 fermenter with a working volume of 3 l and an air flow of  $500 \text{ ml min}^{-1}$ . This possessed a temperature and pH controller, stirring and aeration units (Fig. 2).

The transition from aerobic to anaerobic conditions in the same culture was monitored by the dissolved oxygen unit. The amount of dissolved oxygen was regulated by lowering the amount of air and carbon dioxide flowing into the vessel and increasing the nitrogen flow, making a total gas flow of about  $250 \text{ ml min}^{-1}$ . The flow rates of all three gases were monitored on gas flow meters and were kept constant for each level of dissolved oxygen. On complete anaerobiosis the air and carbon dioxide and nitrogen flows were replaced by  $5\% \text{ CO}_2$  in nitrogen, and all conditions were made anaerobic. At this point the culture changed from aerobic thiosulphate-limited to anaerobic nitrate-limited. The experiment was later repeated to change from aerobic thiosulphate-limited to anaerobic thiosulphate limited. The reverse from anaerobic nitrate-limited to aerobic thiosulphate-limited was also carried out.

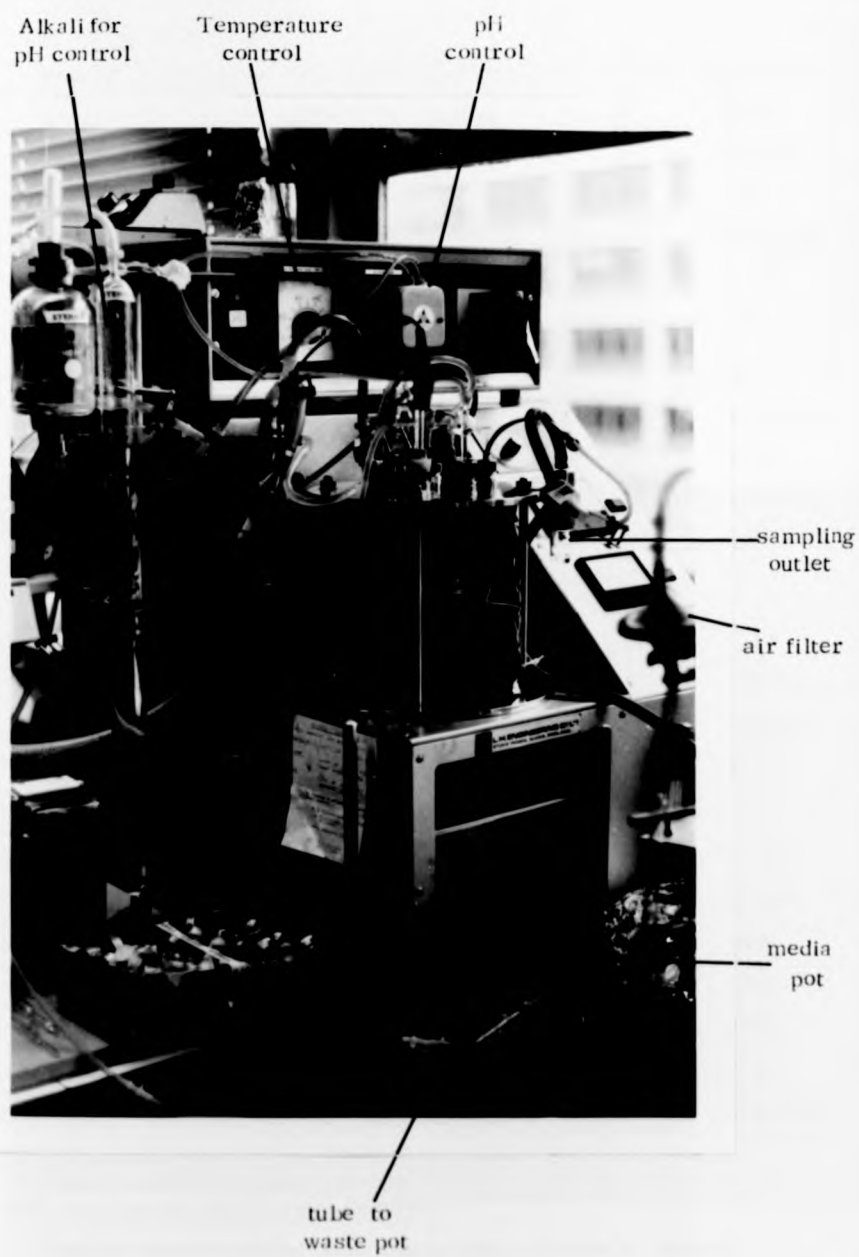
#### 2.2.4 Purity of steady state cultures

The microbiological purity of steady state cultures was checked at weekly intervals by plating  $10^{-5}$  dilution of cultures on:

- (i) agar medium of the same composition as the liquid culture (with  $\text{NaHCO}_3$ ) using  $15 \text{ g l}^{-1}$  Difco Bacto Agar,
  - (ii) agar medium as in (i) supplemented with  $0.1\%$  (w/v) glucose and  $0.65\%$  (w/v) nutrient broth,
  - (iii) agar with  $0.1\%$  (w/v) glucose and  $0.65\%$  (w/v) nutrient broth only.
- The composition of the nutrient broth ( $\text{g l}^{-1}$  solution): 'Lab-Lemco' beef extract, 0.5; yeast extract (oxoid L20), 1; Peptone (oxoid L37), 2.5; and NaCl, 2.5.

#### 2.2.5 Nitrogen source

The nitrogen used to produce anaerobic conditions was obtained from the boil off of a nitrogen liquefaction plant. The oxygen and moisture content are superior to the commercially available white spot nitrogen.



### 2.3 Bacterial composition

#### 2.3.1 Elemental analysis

Four or five litres of cells were harvested from the waste vessel by centrifuging at 10000 rpm for 15 minutes at 4°C in a Beckman model J-21B centrifuge or a MSE model high speed 18 centrifuge. The pellet was resuspended in distilled water and recentrifuged. The final pellet was dried at 105°C and analysed for carbon, hydrogen and nitrogen in a Perkin-Elmer elemental analyser.

#### 2.3.2 G/C ratios

Wet cells collected as before were analysed for their G/C ratio. The G/C ratio in the DNA was determined by the buoyant density estimation method described by Tuovinen *et al.*, (1978). Cells were lysed with sodium lauryl sarcosinate. The DNA was separated from the cell debris by centrifuging at 120 000 g for 36 h in a linear CsCl gradient, and the fractions dialyzed overnight at 4°C against saline citrate.

#### 2.3.3 Electron microscopy

Washed cells suspended in distilled water were prepared for electron microscopic examination on formvar coated grids with either 1% uranyl acetate or by shadowing with gold. Photographs were taken of the observed thiobacilli.

### 2.4 Collection of gases from chemostat

The effluent gas from a thiosulphate-limited anaerobic culture was passed through a U-tube packed with 1/8 inch molecular sieve beads, which increased the surface area, and cooled in liquid nitrogen. The condensed gases were revapourized and analysed for NO and N<sub>2</sub>O by gas chromatography. A Pye Unicam 104 Gas Chromatogram with a thermal conductivity detector was used. The column length was 2.5 m run at a temperature of 50°C and with helium as the carrier gas.

### 2.5 Analysis of steady state cultures

Samples were removed from steady state cultures at regular intervals for chemical and microbiological analysis.

### 2.5.1 Biomass

The optical density of a 4 ml sample taken from the chemostat was read at  $A_{460}$  on a Unicam ultraviolet spectrophotometer 1700. From this reading the dry weight was determined by using a previously prepared calibration curve of dry weight vs. optical density of cells suspended in phosphate solution at pH 7.0. Dry weights were also calculated directly after centrifuging the sample and drying the washed pellet to a constant weight at  $105^{\circ}\text{C}$ . The dry weight readings obtained by both methods were found to be similar; subsequently only the optical density readings were used. It was found that an optical density of 0.500 at 460 nm was equivalent to 130 mg dry wt.  $\text{l}^{-1}$  in anaerobic culture and was 175 mg dry wt. in aerobic culture (Fig. 3).

### 2.5.2 Protein

For the estimation of protein, organisms from 4 ml samples were centrifuged in a Baird and Tatlock Microangle centrifuge at mark 6, washed with distilled water, recentrifuged and finally heated in a boiling water bath for 10 minutes after adding 2.5 ml 0.5 M NaOH. The solubilised protein was then determined (Lowry et al., 1951). 4.5 ml of the reaction mixture (100 ml of 2% (w/v) sodium carbonate in 0.1 M NaOH, 1.5 ml 0.5% (w/v) copper sulphate solution, and 1.5 ml 1% (w/v) potassium sodium tartrate) was added to a final volume of 1 ml 0.5 M NaOH containing the boiled sample of about 50-70  $\mu\text{g}$  protein, mixed and left at room temperature for 10 min. 1 ml of fresh Folin and Ciocalteu's phenol solution (1 part Folin and Ciocalteu's phenol and 1.5 parts water) was added, mixed and after 30 min absorbance was read at 710 nm on a colorimeter. A standard solution of 0.01% <sup>(w/v)</sup> albumin from bovine serum in 0.5 M NaOH was used.

Tests were carried out to examine the possible interference of thio-sulphate in this assay. It was found that 5 mg thiosulphate decreased the expected reading for protein (Fig. 4). The possible interference of the protein determination by any residual thiosulphate was overcome by washing the cells. Sulphur was also found to interfere. This was overcome by differential centrifugation, thus separating the precipitated sulphur from the cells.

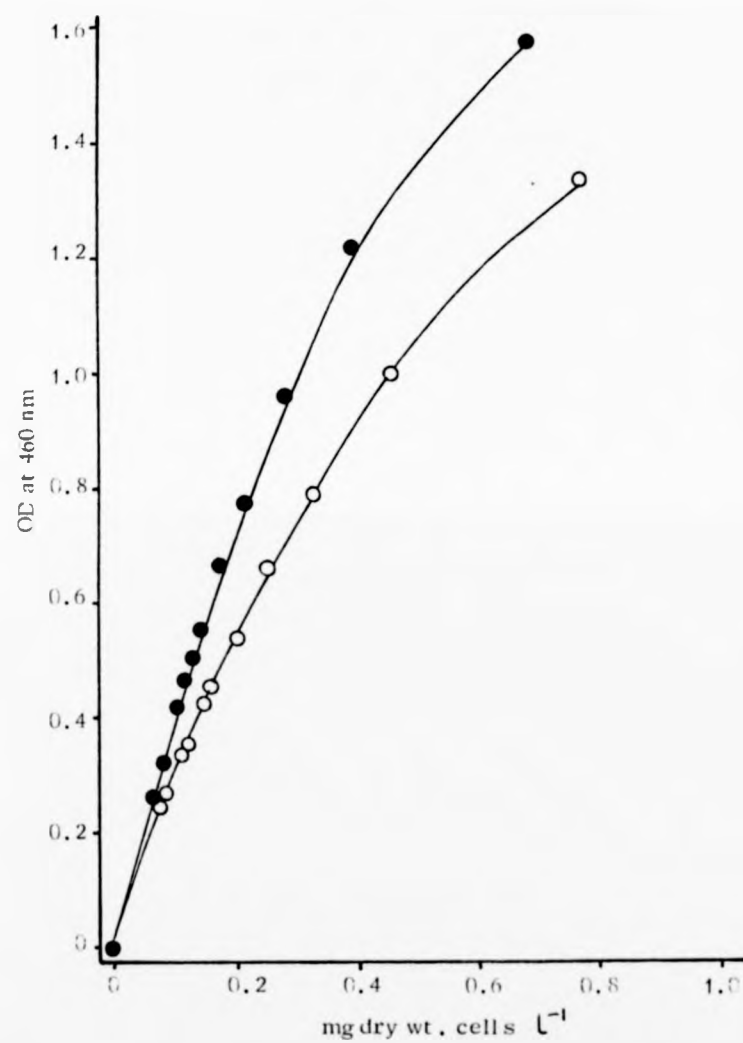


FIG. 3 Comparison of calibration curves of optical density vs. dry weight between aerobic thiosulphate-limited (O) and anaerobic nitrate-limited cultures (●).

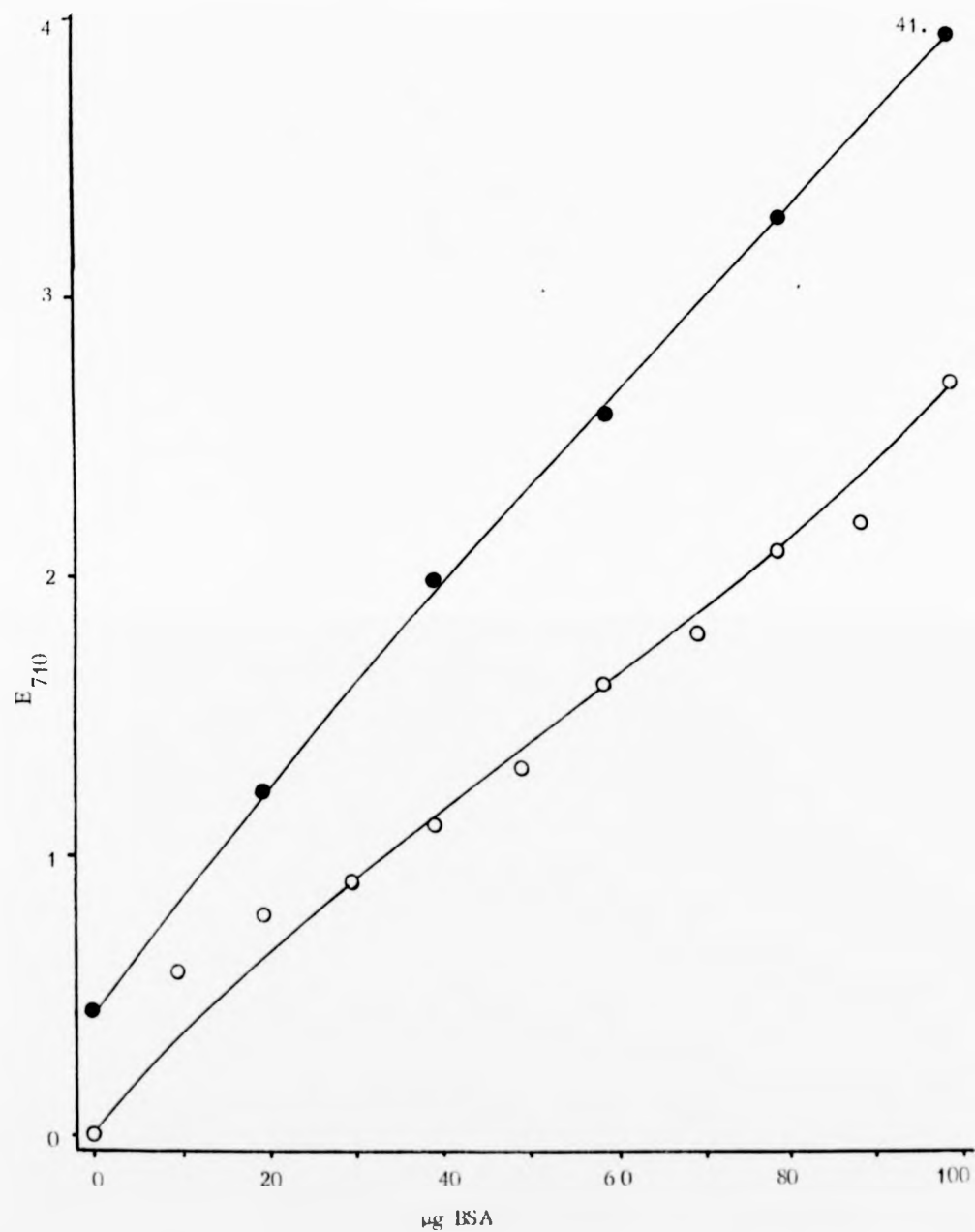


FIG. 4 Interference of thiosulphate in protein assay.

- (●) Albumin from bovine serum (BSA) with 5 mg  $\text{Na}_2\text{S}_2\text{O}_3$   
 (○) Albumin from bovine serum (BSA) only

### 2.5.3 Polythionates

Supernatant liquids from culture samples were analysed for their content of thiosulphate, trithionate and tetrathionate (Kelly, Chambers and Trudinger, 1969). Calibration curves for each reaction were obtained and found to be similar in all cases (Table 1), so one calibration was produced for all polythionates. The reaction was carried out in 25 ml volumetric flasks, each containing 4 ml 'koh' buffer (50 ml 0.2 M sodium dihydrogen orthophosphate and 39 ml 0.2 M sodium hydroxide) and 5 ml 0.1 M potassium cyanide. For the calibration curve 0-7  $\mu\text{mol}$  potassium thiocyanate was added in a final volume of 6 ml (similar with the test sample, omitting the potassium thiocyanate). The flasks were divided into three groups where C was boiled for one hour, after which 1.5 ml 2.5% (w/v) copper sulphate was added; B was not boiled, but the copper sulphate was also added. A was neither boiled nor was copper sulphate added. After 5 min, 3 ml ferric nitrate reagent (303 g  $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$  dissolved in 217.4 ml 72% perchloric acid and 10-15 ml water, and made up to 500 ml with water) was added and the absorbance read at once at 470 nm on a colorimeter. Tetrathionate was read directly from flask A; thiosulphate results from flask B-2A and trithionate was C-B.

A comparison between the cyanolytic method and standard iodine titrimetric means of determining thiosulphate was carried out. Both methods gave the same results. For the whole of this work the cyanolytic method was employed.

### 2.5.4 Sulphate

Sulphate was determined in the supernatant liquid of the culture sample by a turbidity reaction (Gleen and Quastel, 1953). 5 ml of 20% (v/v) glycerol in water was added to 2 ml of liquid containing 1-10  $\mu\text{mol}$  sulphate. 1 ml of 10% (w/v) barium chloride in 5% (v/v) hydrochloric acid added to this caused a precipitation of barium sulphate. The final volume was made up to 10 ml with distilled water. After shaking the mixture, the turbidity was measured at 460 nm on a Corning colorimeter.



TABLE 1 Comparison of treatment A, B, C on thiocyanate to produce calibration curves for the cyanolytic assay

$\mu\text{mol SCN}^-$	Flasks : Treatments		
	A	B	C
	(No $\text{CuSO}_4$ )	(+ $\text{CuSO}_4$ )	(boiled 1 h, + $\text{CuSO}_4$ )
0	0	0	0
1	1.25	1.37	1.42
3	3.55	3.65	3.6
5	5.75	5.7	5.7
7	7.8	7.8	7.8

#### 2.5.5 Sulphur

The presence of any sulphur in the culture was removed by centrifuging a sample in a Baird and Tatlock microangle centrifuge at mark 2. The sulphur precipitated, leaving the cells in suspension. The supernatant was centrifuged again, and the total precipitated sulphur was collected, washed and respun. This sulphur was dissolved by acetone at  $30^\circ\text{C}$  after several hours. Sulphur in a sample of the solution was then determined (Bartlett and Skoog, 1954). 15 ml 0.1% (w/v) potassium cyanide in acetone solvent (19 parts acetone: 1 part water) was added to a 5 ml sample of the solution in a 25 ml volumetric flask, mixed and allowed to stand for 2 min. It was made up to 25 ml with acetone solvent. To a 5 ml aliquot, 5 ml 0.4% (w/v) ferric chloride in acetone solvent was added and mixed. Absorbance was determined immediately at 470 nm in a colorimeter. 5 ml 'flowers' of sulphur dissolved in 100 ml acetone was used as a standard. Sulphur was found to dissolve in neither toluene nor petroleum ether.

The possible interference of thiosulphate, nitrate and nitrite on the assay of elemental sulphur indicated only nitrate (20 mM) as a possible agent, reducing the reaction by 23%. To overcome this, the sulphur was well washed beforehand and a check of the nitrate level was carried out.

### 2.5.6 Nitrite

Nitrite in the supernatant fraction of the culture sample was determined by the Griess-Ilosvay method. Samples containing up to 0.3  $\mu\text{mol}$  nitrite were mixed with 1 ml 0.3 M cadmium sulphate and 1 ml reagent (equal volumes of 0.7% (w/v) sulphanilic acid in 30% (v/v) acetic acid, and of 0.1 g  $\alpha$ -naphthylamine boiled in 20 ml water, then supplemented with 150 ml 30% (v/v) acetic acid), and made up to a final volume of 10 ml with water. After 25 min the absorbance was read at 520 nm on a colorimeter.

Thiosulphate was found to interfere greatly with the nitrite assay. This was overcome by the addition of 0.3 M cadmium sulphate (Table 2). To standardise the reaction, the total thiosulphate concentration was made the same by supplementing the samples with thiosulphate to give a total concentration of about 12 mM. All calibration curves were prepared with 12 mM thiosulphate and 1 ml 0.3 M cadmium sulphate addition. The effect of the concentration of cadmium sulphate was investigated up to 900  $\mu\text{mol}$  and was found to be negligible above 300  $\mu\text{mol}$ .

TABLE 2 Demonstration of thiosulphate interference with nitrite assay and the reversed effect of cadmium sulphate

$\mu\text{mol}$ $\text{NaNO}_2$	$\mu\text{mol}$ $\text{Na}_2\text{S}_2\text{O}_3$	$\mu\text{mol}$ $\text{CdSO}_4$	$A_{520}$
0	0	0	0
0	12.09	300	0
0.15	0	0	5.1
0.15	12.09	0	1.7
0.15	0	300	5.3
0.15	12.09	300	4.7

### 2.5.7 Nitrate

Nitrate (0.03 to 3 mg) was determined by titration with 1.67 mM potassium dichromate, using 0.025 M ferroin as indicator (Kolthoff and Belcher, 1957).

A great deal of time was spent in finding a method to analyse nitrate that would be free from interferences from thiosulphate. The first method involved the addition of 0.5 ml concentrated phenol-disulphonic acid (25 g colourless phenol dissolved in 150 ml concentrated sulphuric acid) to the nitrate sample, followed by 5 ml 7 M sodium hydroxide. The final volume of 25 ml was made up with ice cold water. After 10 minutes the absorbance was read at 420 nm. The length of time for the colour to develop was increased to enable a better colour to evolve, but with no effect.

In the second method, the nitrate sample was made up to 10 ml in a 100 ml beaker to which 75 mg calcium carbonate was added. The mixture was evaporated to dryness, cooled and 2 ml phenol-disulphonic acid added. After leaving for 10 minutes, 20 ml ice cold water was added and left a further 10 minutes. 20 ml of 1:1 ammonium hydroxide:water solution was added, and the solution poured into 100 ml volumetric flasks which were made up to 100 ml by ice cold distilled water. The absorbance was read as before. The increase in time for colour development had no effect.

The third method (Snell and Snell, 1959) was very similar to the previous method, except that a mixture of 1:2 ammonium hydroxide:water was used, and the final volume was 50 ml. This last method seemed to be the most efficient where the readings were in proportion with the quantity of nitrate present. When culture samples were assayed, it became evident that the presence of thiosulphate interfered greatly with the reaction.

A fourth method was investigated (BSP Handbook, 'Chemical Analysis of Fresh Water'). This involved the conversion of nitrate to nitrite which was finally analysed by the Griess-Ilosvay reagent. The  $\text{NO}_3\text{-N}$  ranging from  $1\text{ }\mu\text{g ml}^{-1}$  to  $400\text{ }\mu\text{g ml}^{-1}$  was made up to 40 ml with distilled water in boiling tubes. 1 ml of 1 M sodium hydroxide and 1 ml reaction mixture (5 ml of 0.39%  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  and 25 ml of 1.2 g hydrazine sulphate in 250 ml water, made up to 50 ml with double distilled water) was added to the tubes and mixed. After incubation at  $30^\circ\text{C}$  for 30 minutes, 0.5 ml acetone was added. After a further 5 minutes, 1 ml 1 M HCl was added and mixed.

The mixture was left overnight before testing for nitrite as before.  $10 \mu\text{mol NO}_3\text{-N}$  was found to be the maximum that could be analysed, giving a standard range of  $0.01\text{--}0.12 \mu\text{mol}$  nitrite. This reaction was modified by using 50 ml volumetric flasks rather than boiling tubes. The different concentrations of nitrate showed an increase in nitrite in proportion to the volume of the sample taken (Fig. 5). The effect of thiosulphate on this assay was also investigated and was found to inhibit completely the reaction, *i.e.* 1 and  $2 \mu\text{mol}$  nitrate gave a reading of 1.25 and 2.4. The addition of 0.1%, 0.25%, 0.5% and 1% thiosulphate decreased these readings to 0.02. This method was modified by increasing the reduction mixture or the alkali or acid. These modifications were found to decrease the reactivity and so were abandoned.

The addition of concentrated sulphuric acid or liquid bromine was investigated in an attempt to remove the thiosulphate from the mixture of thiosulphate and nitrate. 1 ml concentrated sulphuric acid was added to a mixture of 0.2 mg nitrate and 0.1% <sup>(w/v)</sup> thiosulphate. After 15 minutes the mixture was heated for 30 minutes. Method three was then employed on the remaining liquor with no improved response. Alternatively, 1 drop of liquid bromine was added to the mixture and after 15 minutes it was heated to drive off the residual bromine. The analysis of the nitrate indicated that this was successful, *i.e.* nitrate alone and a mixture of thiosulphate and nitrate gave the same reading after treatment, but these results were not consistent.

A fifth and final method was successfully carried out (Kolthoff and Belcher, 1957). The thiosulphate still interfered, but this was overcome by maintaining a constant level of 12 mM thiosulphate. The increase of thiosulphate caused an increase in the reaction in proportion to the nitrate concentration (Fig. 6). Approximately 0.2 g sodium bicarbonate was added to a mixture of 5 ml 0.02 N ferrous sulphate solution in 50% (v/v) sulphuric acid, and 5 ml concentrated sulphuric acid in 50 ml conical flasks, and cooled. The solution of nitrate and thiosulphate in a volume of

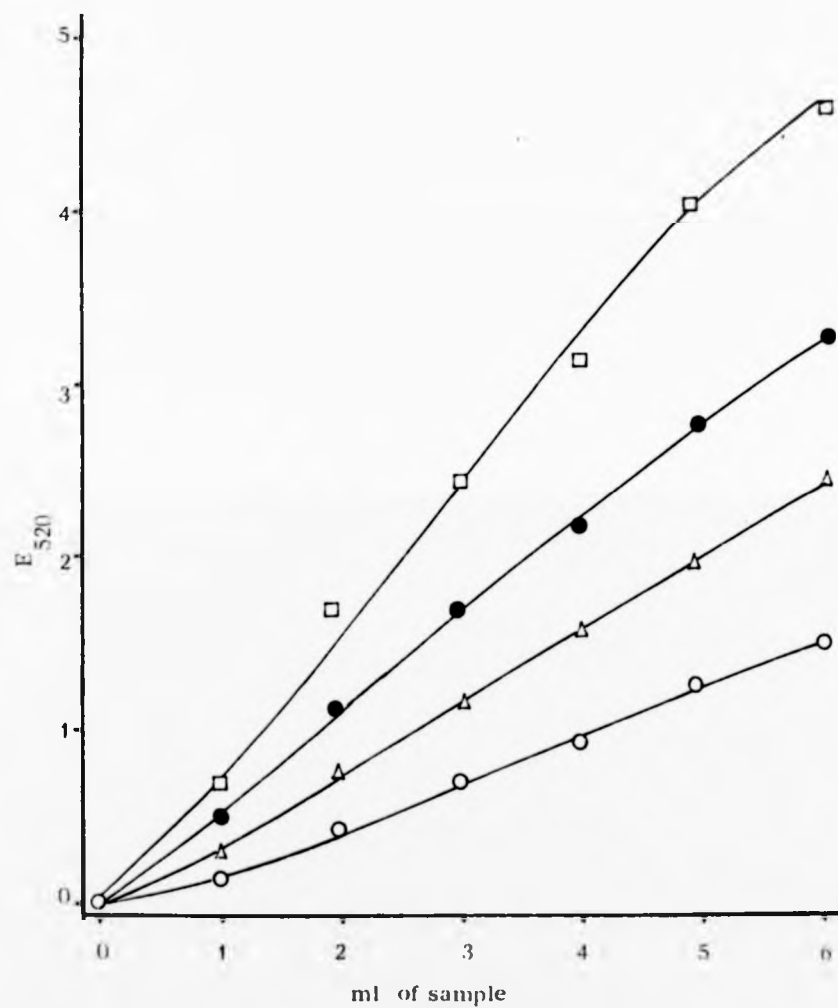


FIG. 5 Nitrite production from nitrate proportional to sample taken;  $0.08 \mu\text{mole NO}_2^- \text{ml}^{-1}$  ( $\square$ ),  $0.04 \mu\text{mole NO}_2^- \text{ml}^{-1}$  ( $\bullet$ ),  $0.02 \mu\text{mole NO}_2^- \text{ml}^{-1}$  ( $\Delta$ ),  $0.01 \mu\text{mole NO}_2^- \text{ml}^{-1}$  ( $\circ$ ).

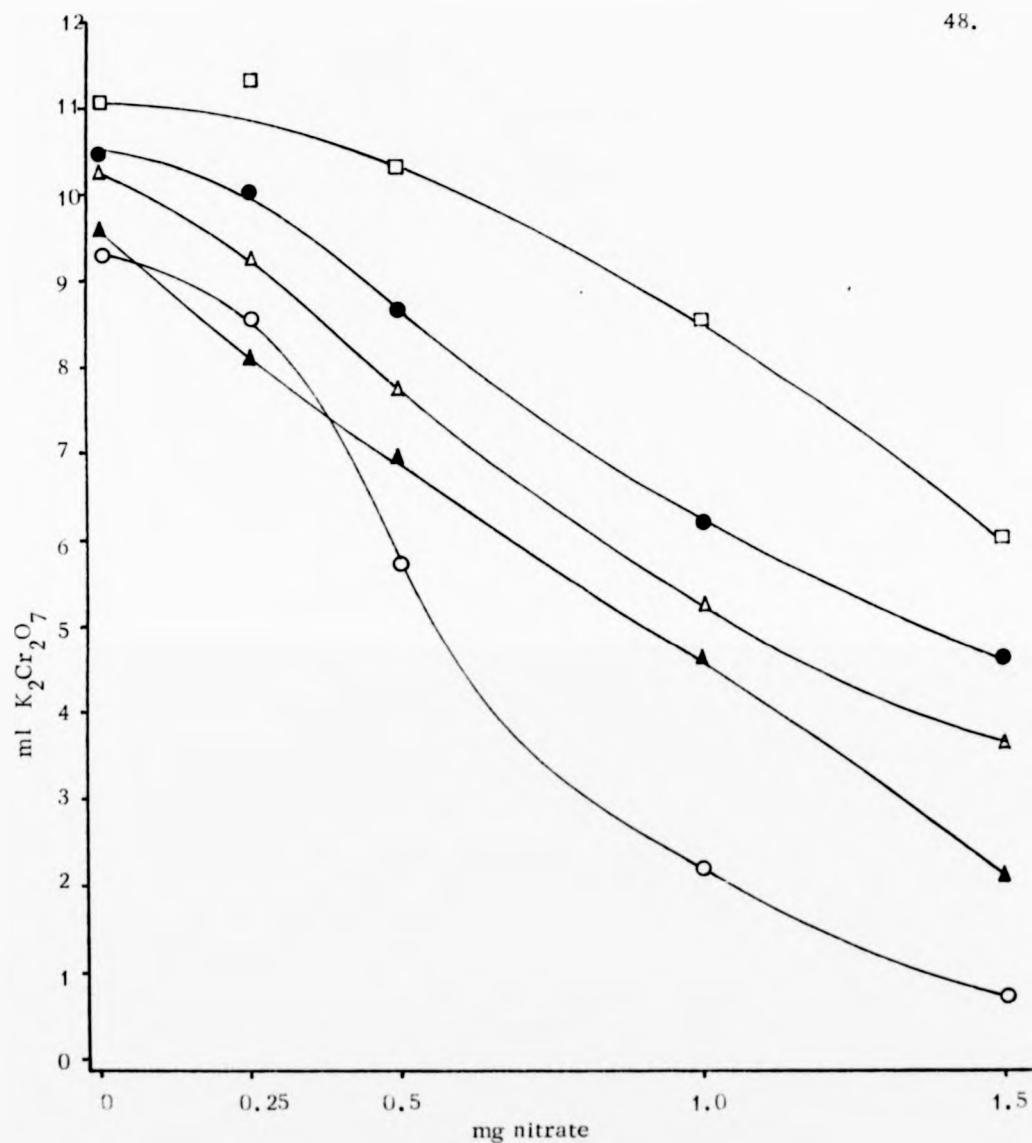


FIG. 6 The effect of thiosulphate concentrations on the Kolthoff and Belcher (1957) nitrate assay; 40.29  $\mu\text{mole}$  (□), 20.14  $\mu\text{mole}$  (●), 10.07  $\mu\text{mole}$  (Δ), 2.02  $\mu\text{mole}$  (▲), 0  $\mu\text{mole}$  (○).

5 ml and approximately 0.5 g sodium chloride was added. The flasks were put in a boiling water bath for 5 min, where the solution changed from a brownish violet to a clear yellow. The flasks were cooled and 1 ml of 90% <sup>(v/v)</sup> (S.G. 1.75) phosphoric acid added. Two drops 0.025 M ferroin (o-phenanthroline) solution was added, and titrated with 0.01 N potassium dichromate to the end point from orange red to blue green. The amount of sodium bicarbonate and sodium chloride was not critical.

The presence of nitrite did not interfere with any of the methods attempted.

#### 2.5.8 Glycolate

The possible production of glycolate in the chemostat by *T. denitrificans* was investigated. 2 ml of 0.01% <sup>(v/v)</sup> 2,7-dihydroxynaphthalene was added to a total volume of 0.2 ml of 2-20  $\mu$ mol glycolate in 2 N sulphuric acid standing in ice. The mixture was stirred and boiled for 20 minutes. After returning to the ice, 4 ml 2 N sulphuric acid was added, and after cooling was read at 530 nm. The effect of nitrate substantially interfered with the reaction, i.e. 0.1 mg potassium nitrate decreased the intensity by 50%. The solution became yellow instead of the expected pink. Nitrite decreased the activity by 20% and thiosulphate by 7%. There was no way possible to reduce the interference of all three compounds. An attempt to find a wavelength by which a mixture of nitrate and glycolate could be measured was unsuccessful. This resulted in abandoning any attempt at analysing the medium for the possible presence of glycolate. The presence of excreted organic matter was found negligible in the *T. denitrificans* culture of Timmer-ten Hoor (1976).

### 2.6 Enzyme analysis

#### 2.6.1 Preparation of crude extract for enzyme assays

Organisms were harvested from steady state chemostats by centrifuging at 4°C in a Beckman model J-21B centrifuge or a MSE model high speed 18 centrifuge at 10,000 rpm for 15 minutes. The pellet was resuspended in distilled water and recentrifuged. The final pellet was either stored at -20°C or was used immediately for enzyme assay.

The organisms were suspended (25% v/v) in 0.05 M-Tris(hydroxymethyl)methylamine/HCl buffer (pH 7.54) and passed twice through an

Amino French pressure cell at 140 MPa. The broken cell suspension was centrifuged at 11,500 rpm for 30 minutes at 4°C, and the supernatant liquid was used for enzyme assays.

#### 2.6.2 Nitrate reductase

Nitrite production from nitrate was measured, using NADH as electron donor (Sawhney and Nicholas, 1977) in reaction mixtures in open test tubes containing (final volume 2 ml): Tris/HCl, pH 7.54, 80 µmol; phenazine methosulphate, 1 µmol; KNO<sub>3</sub>, 1 µmol; crude extract, 0.01 ml. After storing this mixture at 4°C for 15 minutes, it was equilibrated at 30°C for 2 minutes and the reaction initiated by adding 1 µmol NADH. After 15 minutes the reaction was terminated by adding 0.1 ml 1 M-acetaldehyde and 0.03 mg alcohol dehydrogenase in 0.1 ml 0.05 M Tris/HCl, pH 7.54, to oxidize the residual NADH. Nitrite was determined by the Griess-Ilosvay method (previously described in section 2.5.6). Activity was expressed as nmol NO<sub>2</sub><sup>-</sup> produced min<sup>-1</sup> (mg protein)<sup>-1</sup>. A calibration curve using 0-0.1 µmol sodium nitrite was produced.

Two very similar methods were used at first (Adams, Warnes and Nicholas, 1971; Sawhney and Nicholas, 1977) with the latter being the most successful. The former method did not produce any results. This was thoroughly investigated, which included the possible interference of any of the components of the enzyme assay into the Griess-Ilosvay assay. The following 0.05 M phosphate buffer containing 0.2 mM EDTA (pH 7.0), 2 µmol NADH, 1 M acetaldehyde and alcohol dehydrogenase suspension were individually omitted from the reaction mixture on which the nitrite assay was performed. The absence of NADH had the least effect, whereas the omission of acetaldehyde and alcohol dehydrogenase almost completely inhibited the reaction. The activities of NADH and alcohol dehydrogenase were checked, and found to be functioning properly. The NADH-linked enzyme activity of the stored samples was supposed to increase with the addition of 1 µmol glutathione (reduced) or 1 µmol phenazine methosulphate (Sawhney and Nicholas, 1977). Only the latter



was found to do this, and so was always present in the assays (Table 3). The phenazine methosulphate actually depressed the nitrite calibration curve due to its interference with the Griess-Ilvovsay reagent reaction (Fig. 7). The glutathione tended to decrease the enzyme activity. The length of incubation at 30°C was varied from 10 to 30 minutes with no increase in activity after 15 minutes. The increased potassium nitrate concentration from 1-10 µmol showed no proportional increase in activity, hence only 1 µmol KNO<sub>3</sub> was used.

**TABLE 3** Effect of reduced glutathione and phenazine methosulphate on the activity of nitrate reductase from cells stored at -20°C

(Culture grown anaerobically on thiosulphate-limited media with nitrate)

Reduced glutathione (µmol)	Phenazine methosulphate (µmol)	A <sub>520</sub>	µmol NO <sub>2</sub> <sup>-</sup> produced
-	-	0.029	0.008
1	-	0.023	0.006
-	1	0.041	0.030

### 2.6.3 Nitrite reductase

Nitrite reductase activity was measured at 30°C in 10 ml Warburg flasks (Aminuddin and Nicholas, 1973) containing (final volume 1 ml): Tris(hydroxymethyl)methylamine, pH 7.54, 40 µmol; phenazine methosulphate, 1 µmol; sodium nitrite, 0.6 µmol; crude extract, 0.01 ml. The reaction was initiated by adding 1 µmol NADH, after flushing the flasks with oxygen-free nitrogen for 10 minutes. The reaction was terminated after 0, 3, 6 and 10 minutes as for nitrate reductase assays, and nitrite was determined. Activity was expressed as nmol NO<sub>2</sub><sup>-</sup> reduced min<sup>-1</sup> (mg protein)<sup>-1</sup>.

Attempts to carry out this assay in test tubes proved unsuccessful as an anaerobic condition was found to be essential. Different concentrations of enzyme extract, 0.01, 0.05 and 0.1 ml were used,

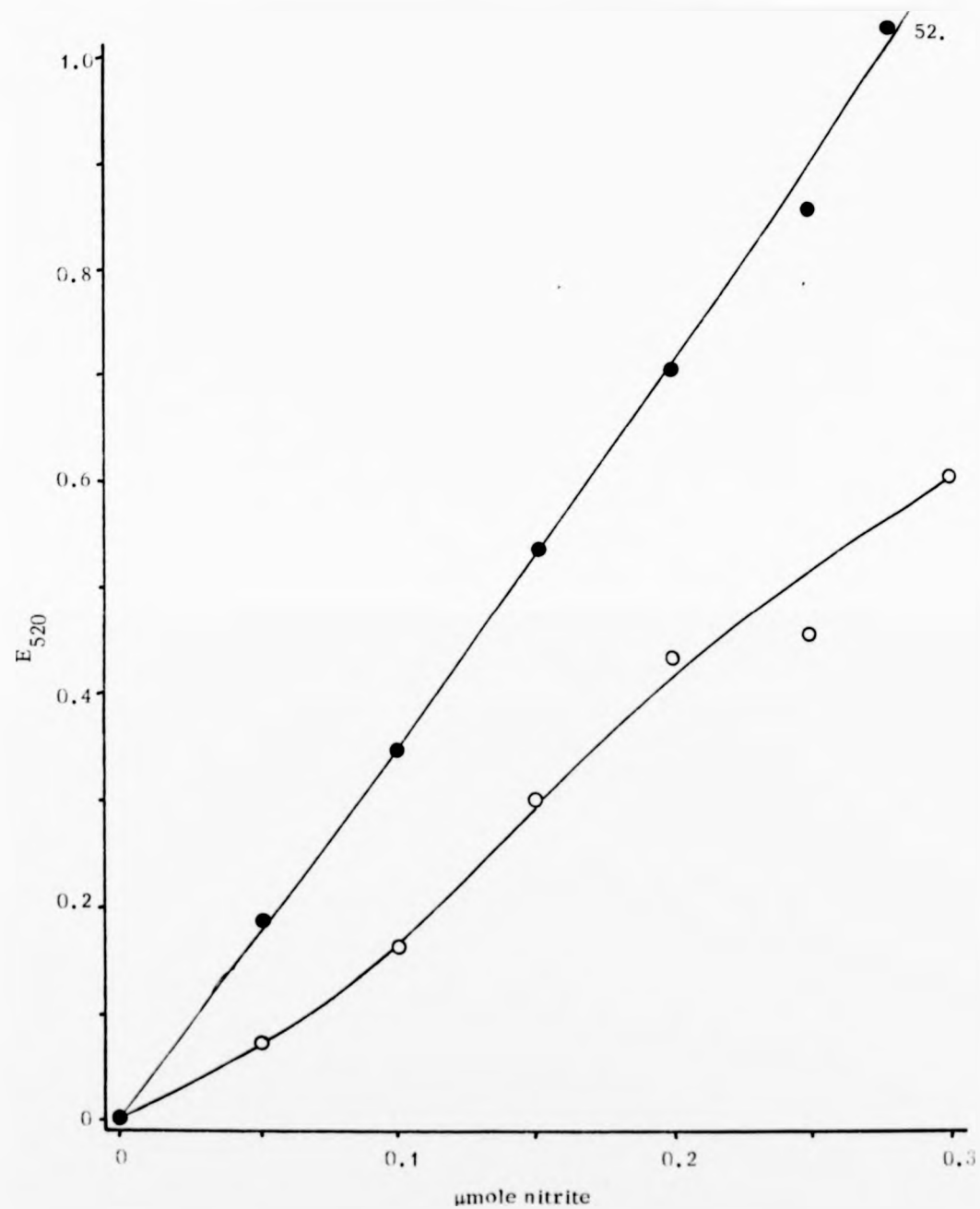


FIG. 7 Comparison of calibration curve of nitrite for nitrate reductase enzyme with (○) and without phenazine methosulphate (●).

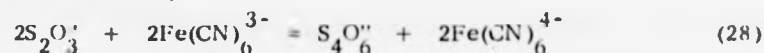
but the 0.05 and 0.1 ml were found to make the reaction mixture cloudy and so interfere with the colorimetric reading. A calibration curve was produced using sodium nitrite 0-0.8  $\mu\text{mol}$ . In the original paper (Aminuddin and Nicholas, 1973), 2  $\mu\text{mol}$  NADH was used with 0.1 ml 0.1 M acetaldehyde and 0.1 ml of 0.03 mg alcohol dehydrogenase in buffer as terminator. With this mixture no results were obtained, hence 1  $\mu\text{mol}$  NADH was used as in the nitrate reductase assay. This led to a positive result. Originally, even by increasing the acetaldehyde and alcohol dehydrogenase concentrations, there was no effect on removing the residual NADH interference.

A different method of nitrite assay was attempted, using 1% (w/v) sulphanilamide in 1 M HCl and  $\alpha$ -naphthylamine solution, but with no effect.

#### 2.6.4 Thiosulphate-oxidizing enzyme

Ferricyanide reduction (Trudinger, 1961) was measured spectrophotometrically with a reaction mixture in 1 cm cuvettes containing (final volume 3 ml): potassium phthalate buffer, pH 5.0, 300  $\mu\text{mol}$ ; sodium thiosulphate, 30  $\mu\text{mol}$ ; potassium ferricyanide, 3  $\mu\text{mol}$ ; crude extract, 0.05 ml. Thiosulphate and ferricyanide were omitted from the reference cuvette. The reaction was initiated by adding the extract. Activity was measured for up to 60 minutes and expressed as nmol ferricyanide reduced  $\text{min}^{-1}$  ( $\text{mg protein}^{-1}$ ). An alternative reaction mixture was investigated with a potassium phosphate buffer at pH 7.4 replacing the potassium phthalate buffer (Lyric and Suzuki, 1970). A calibration curve with both buffers was produced, using 0-3  $\mu\text{mol}$  potassium ferricyanide.

Reaction involved:



Any activity recorded with the buffer above was taken into account.

#### 2.6.5 Sulphur-oxidizing enzyme

Sulphur oxidizing enzyme was assayed by measuring thiosulphate production and oxygen uptake using Warburg manometers (Suzuki and Silver, 1966).

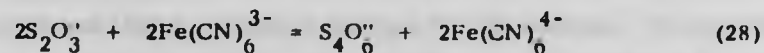
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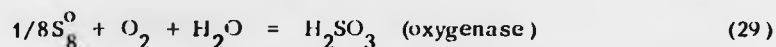
Reaction involved:



Any activity recorded with the buffer alone was taken into account.

#### 2.6.5 Sulphur-oxidizing enzyme

Sulphur oxidizing enzyme was assayed by measuring thiosulphate production and oxygen uptake using Warburg manometers (Suzuki and Silver, 1966).



The reaction mixtures contained (final volume 2 ml): Tris(hydroxymethyl)-methylamine/HCl, pH 7.8, 500  $\mu\text{mol}$ ; sulphur (B.D.H. 'Optran' grade), 48 mg; 2,2'-bipyridyl, 0.2  $\mu\text{mol}$ ; catalase, 250  $\mu\text{g}$ ; crude extract (fresh and stored), 0.1 ml. The reaction in air filled flasks was initiated by adding 5  $\mu\text{mol}$  reduced glutathione. After 210 minutes, during which oxygen uptake was recorded, flask contents were sampled and thiosulphate was determined cyanolytically as ferric thiocyanate (Suzuki, 1965a).

The ability of T. denitrificans extracts to oxidise sulphur in the absence of reduced glutathione was investigated.

## 2.7 Anaerobic nitrite and nitrate reduction by suspensions of T. denitrificans

From steady state cultures held at different dissolved oxygen concentrations, 200 ml samples were aseptically removed and the organisms harvested by centrifuging, washed and resuspended in 40 ml salt solution (section 2.2.1) lacking thiosulphate and nitrate or nitrite. This suspension was divided equally into four universal bottles, with two additional bottles containing salts solution only. These were all sealed with suba-seal vaccine stoppers, made anaerobic by passage of oxygen-free nitrogen for 5 minutes, and incubated at 30°C.  $\text{Na}_2\text{S}_2\text{O}_3$  (2  $\mu\text{mol ml}^{-1}$ ) and  $\text{NaNO}_2$  (1.8  $\mu\text{mol ml}^{-1}$ ) were added to two universal bottles containing the suspended cells and to one with salts solution only.  $\text{Na}_2\text{S}_2\text{O}_3$  (2  $\mu\text{mol ml}^{-1}$ ) and  $\text{KNO}_3$  (2  $\mu\text{mol ml}^{-1}$ ) were added to the remaining bottles. Samples were removed for thiosulphate, tetrathionate, nitrate and nitrite analysis at intervals for 300 minutes. The polythionates were assayed immediately. The remaining sample was frozen immediately in dry ice and assayed for nitrate and nitrite the following day.

## 2.8 Effect of 2:4-Dinitrophenol on carbon dioxide fixation

### 2.8.1 Cell suspensions

Organisms grown aerobically and anaerobically were harvested as before by centrifugation, washed and suspended in salts solution lacking thiosulphate and nitrate.

### 2.8.2 Gas exchange

Gas exchange was measured by Warburg manometry at 30° C from single and double armed flasks.

### 2.8.3 Procedure

A series of experiments were carried out to find at what density of organism and concentration of thiosulphate, sulphur, sulphide (and nitrate when anaerobic) was suitable to obtain accurate measurements. The cell suspensions were diluted to give either a dilute suspension (equivalent to about 6 mg protein ml<sup>-1</sup>) or dense suspension (equivalent to about 12 mg protein ml<sup>-1</sup>). For aerobic suspensions, 0.9 μmol thiosulphate and 0.6 μmol sulphide was used; anaerobic suspensions, 0.9 μmol thiosulphate with 6-18 μmol nitrate, and 0.6 μmol sulphide with 4-12 μmol nitrate. Once the concentration of thiosulphate, sulphide and nitrate had been decided upon, the effect of 0, 10<sup>-5</sup>, 10<sup>-4</sup>, 10<sup>-3</sup> M 2,4-dinitrophenol was investigated. Any remaining thiosulphate or polythionates produced were assayed for by the cyanolysis method.

A third series of experiments was carried out with one concentration of 2,4-dinitrophenol (10<sup>-4</sup> M). Any remaining nitrate or thiosulphate, or nitrite and polythionate produced in the anaerobic cultures were assayed for as before.

Finally, one experiment was carried out with aerobically grown cells which had been harvested 15 hours before and maintained shaking in salts solution lacking in thiosulphate and nitrate at 30° C.

With each investigation, the flask acting as the thermobarometer contained 2.3 ml of 0.01% <sup>(-1/100)</sup>mercuric chloride. The total volume of each experimental flask was 2.3 ml of which 2 ml was the cell suspensions in the main chamber; 0.2 ml of thiosulphate or sulphide with or without nitrate in one side arm; 0.1 ml of 0.1 M NaH<sup>14</sup>CO<sub>3</sub> (20 μc/ml) in the second side arm. The flasks were equilibrated when attached to the manometers in the water bath at 30° C for 10 minutes. Aerobically the manometers and flasks were left open to the atmosphere during this time.

Anaerobically the flasks and manometers were flushed with oxygen-free nitrogen for 10 minutes and carefully closed. The manometers were adjusted to measure maximum oxygen uptake when aerobic, or nitrogen gas release when anaerobic. Once the flasks had equilibrated, the  $\text{NaH}^{14}\text{CO}_3$  and sulphur compound (and nitrate) were tipped in. The gas flow was recorded until no manometric change was observed. At this point, samples were taken for polythionate, nitrate, nitrite and sulphate assay.

#### 2.8.4 Carbon dioxide fixation

Carbon dioxide fixation was estimated at the end of the experiment. A 1 ml sample of the suspension of organisms was pipetted into 2 ml of ethanol containing 10% <sup>(v/v)</sup> acetic acid. 0.5 ml of this mixture was put into low phosphate glass scintillation vials and evaporated to dryness using a hair drier. The dried cells were resuspended in a scintillant composed of: 750 ml toluene, 250 ml methanol and 6 g butyl PBD. The  $^{14}\text{C}$  was counted by a Packard 'Tri-carb' Liquid Scintillation Counter 2425.

#### 2.9 Sources of materials

5%  $\text{CO}_2$  <sup>(v/v)</sup> in nitrogen, 5%  $\text{CO}_2$  <sup>(v/v)</sup> in air was supplied by British Oxygen Company, special gases division (Coventry);  $\text{N}_2\text{O}$  research grade G, 99.998% purity supplied by British Oxygen Company, medical department (London).  $\text{CO}_2$  supplied by Air Products Ltd. (James Bridge, Birmingham). Alcohol dehydrogenase, nitrate reductase from *E. coli*, 8 NADH and phenazine methosulphate supplied by Sigma (Kingston-upon-Thames, Surrey). 2,2'-Bipyridyl, phenoldisulphonic acid, potassium tetrathionate and 0.025 M ferroin (o-phenanthroline) solution from British Drug Houses Ltd. (Poole). Toluene supplied by Koch-Light laboratories (Colnbrook, Buckinghamshire). Difco 'Bacto-Agar' from Michigan, U.S.A. All other chemicals came from British Drug Houses Ltd. (Poole), Hopkins and Williams Ltd. (Chadwell Heath, Essex) and Fisons Scientific Apparatus Ltd. (Loughborough).

PART 3: GROWTH PHYSIOLOGY OF THIOBACILLUS  
DENITRIFICANS IN BATCH AND CHEMOSTAT  
CULTURE



### PART 3: GROWTH PHYSIOLOGY OF *THIOBACILLUS DENITRIFICANS* IN BATCH AND CHEMOSTAT CULTURE

#### 3.1 Introduction

*Thiobacillus denitrificans* has been subjected to considerable physiological and biochemical studies largely concerned with batch cultures and the mechanism of sulphur compound oxidation and nitrate reduction (Beijerinck, 1904; Lieske, 1912; Baalsrud and Baalsrud, 1952, 1954; Aubert, Millet and Milhaud, 1959; Woolley, Jones and Happold, 1962; Bowen, Butler and Happold, 1966; Sargeant *et al.*, 1966; Adams, Warnes and Nicholas, 1971; Taylor, Hoare and Hoare, 1971; Aminuddin and Nicholas, 1973, 1974a, b; Schedel, Le Gall and Baldensperger, 1975; Sawhney and Nicholas, 1977, 1978a, b). Only recently has any attempt been made to study the bioenergetics of this organism by means of continuous culture (Justin and Kelly, 1976; Timmer-ten Hoor, 1976).

This study was performed to seek information on the behaviour of continuous cultures of *T. denitrificans* subject to substrate limitation under aerobic and anaerobic conditions and to determine the ease with which adaptation between aerobiosis and anaerobiosis could occur. The results enable calculations of comparative growth yields and 'maintenance energy' requirements under a variety of physiological steady state conditions.

#### 3.2 Batch Cultures

Enrichment cultures of the organism was setup using two soil samples, three mud samples (including a sewage outfall mud), stream water, freshwater stream mud. Only the latter two gave successful enrichments from which a pure culture of *Thiobacillus denitrificans* was obtained, the isolate from the freshwater stream mud being the best denitrifier of the two. However, most of the work on this organism has been carried out with the Torrey collection strain NCIB 9548. This is due to its less slimy characteristic which the other two displayed. All three have been maintained in filled bottle batch culture in which they tend to accumulate sulphur, polythionates and nitrite, and a nitrogen-containing gas is released (Fig. 8a, 8b).

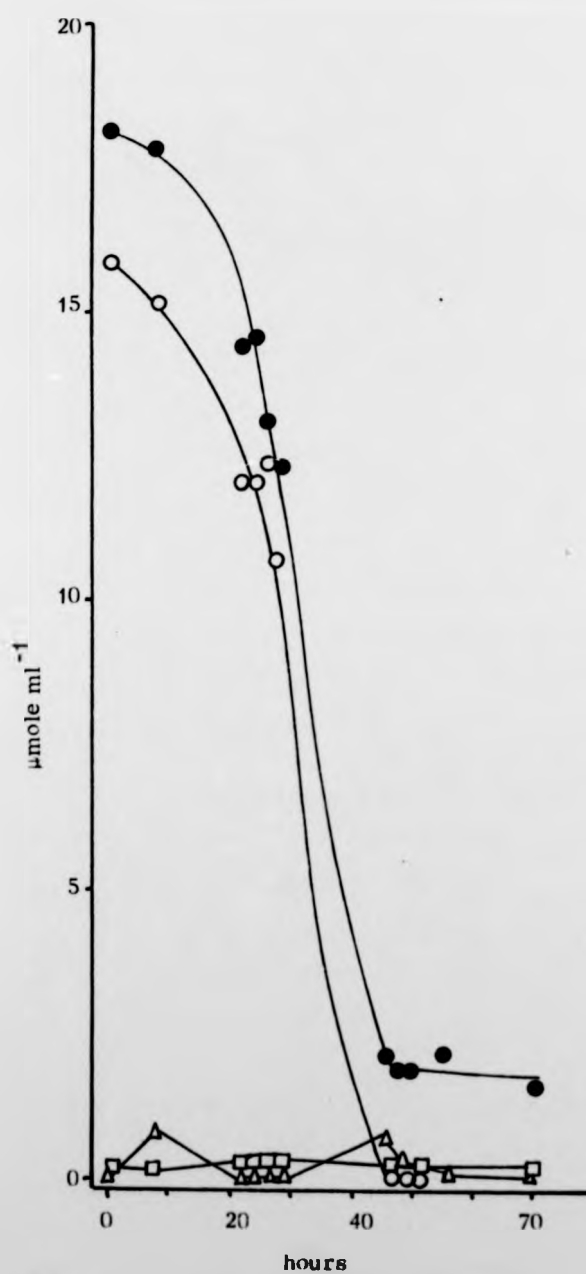


FIG. 8a Thiosulphate, nitrate consumption by *T. denitrificans* from fresh water stream mud; ●,  $\text{S}_2\text{O}_3^{2-}$ ; □,  $\text{S}_4\text{O}_6^{2-}$ ; Δ,  $\text{S}_3\text{O}_6^{2-}$ ; ○,  $\text{NO}_3^-$ .

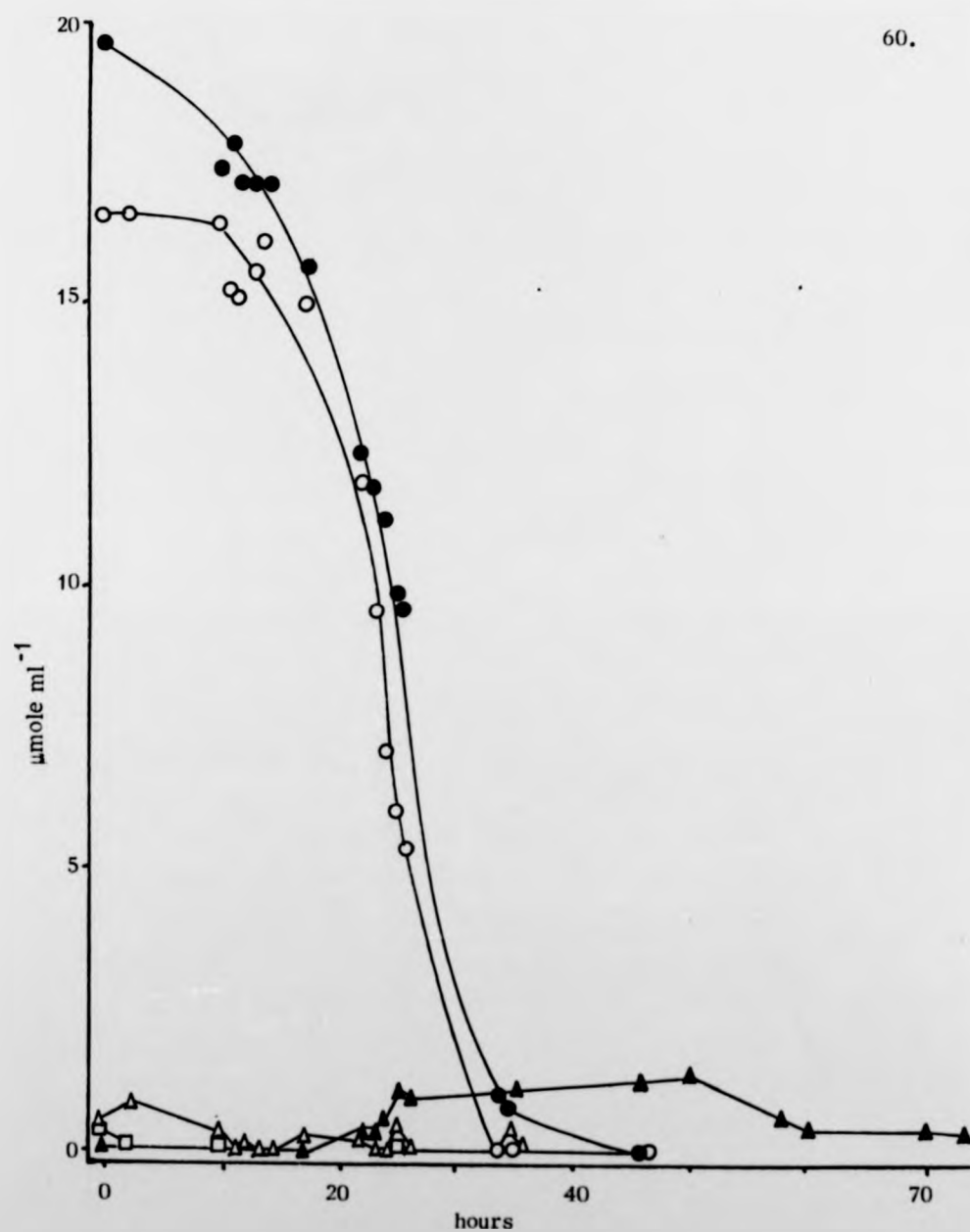
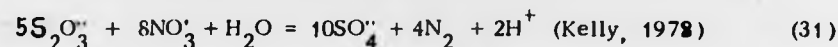


FIG. 8b Thiosulphate, nitrate consumption and nitrite production by *T. denitrificans* strain NCIB 9548:  $\bullet$ ,  $\text{S}_2\text{O}_3^{2-}$ ;  $\square$ ,  $\text{S}_4\text{O}_6^{2-}$ ;  $\Delta$ ,  $\text{S}_3\text{O}_6^{2-}$ ;  $\circ$ ,  $\text{NO}_3^-$ ;  $\blacktriangle$ ,  $\text{NO}_2^-$ .

These three cultures were able to grow anaerobically in conditions where the thiosulphate:nitrate ratio was equal to the theoretical (5:8). i.e.



with excess nitrate (5:16) and with excess thiosulphate (5:2). In the first case all the thiosulphate was oxidized with 15.6% of the original nitrate remaining; in the second, all the thiosulphate was used in the same length of time with 47.43% nitrate remaining. In the third test all the nitrate was reduced in half the time and 69.75% thiosulphate remained (Fig. 9a, b, c). This is not quite in agreement with the theoretical where in the first case no thiosulphate nor nitrate should remain; second, 50% nitrate should be left unreduced; third, 75% thiosulphate should remain. The thiosulphate in all cases is partially oxidized to provide reducing equivalents for carbon dioxide fixation and partly for energetic reasons. The thiosulphate consumed for the energetic reasons would be less than the overall thiosulphate oxidation. Hence the total thiosulphate consumption should be greater than the theoretical nitrate reduction when thiosulphate is not limiting. This may account for the remaining 15.6% nitrate. In the third case more thiosulphate was oxidized than was expected, but trithionate accumulated in some cases which may account for this.

Batch cultures were also established in shake flasks under aerobic conditions in the presence of 20 mM thiosulphate and 22 mM nitrate. A small amount of nitrite (0.03  $\mu\text{mole}$ ) was produced from the nitrate, but other than this the nitrate content remained constant throughout at about 22 mM. All the thiosulphate was used with a slight accumulation of tetrathionate (0.26 mM) and trithionate (0.80 mM) which was completely oxidized after 95 hours, with the accumulation of 0.001 mg sulphur.

At first the medium used in aerobic cultures contained sodium bicarbonate as the carbon source. In the shake flask cultures and chemostat batch cultures this was found to increase the pH of the media to 8.7, due to the release of carbon dioxide out of solution making the medium unsuitable for T. denitrificans to grow. Later the same medium was used without the bicarbonate. This led to a rapid growth with few pH problems with the

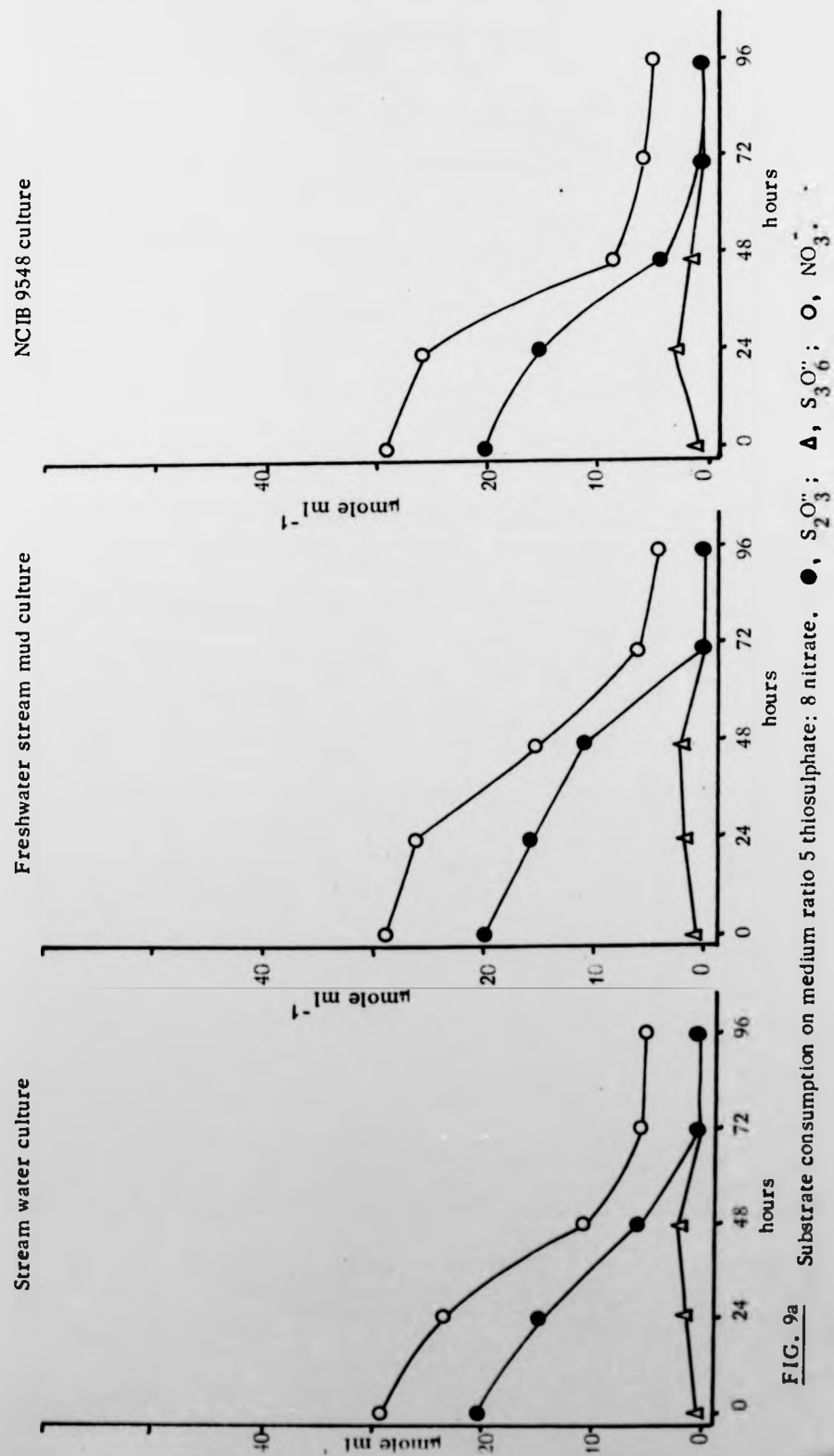


FIG. 9a

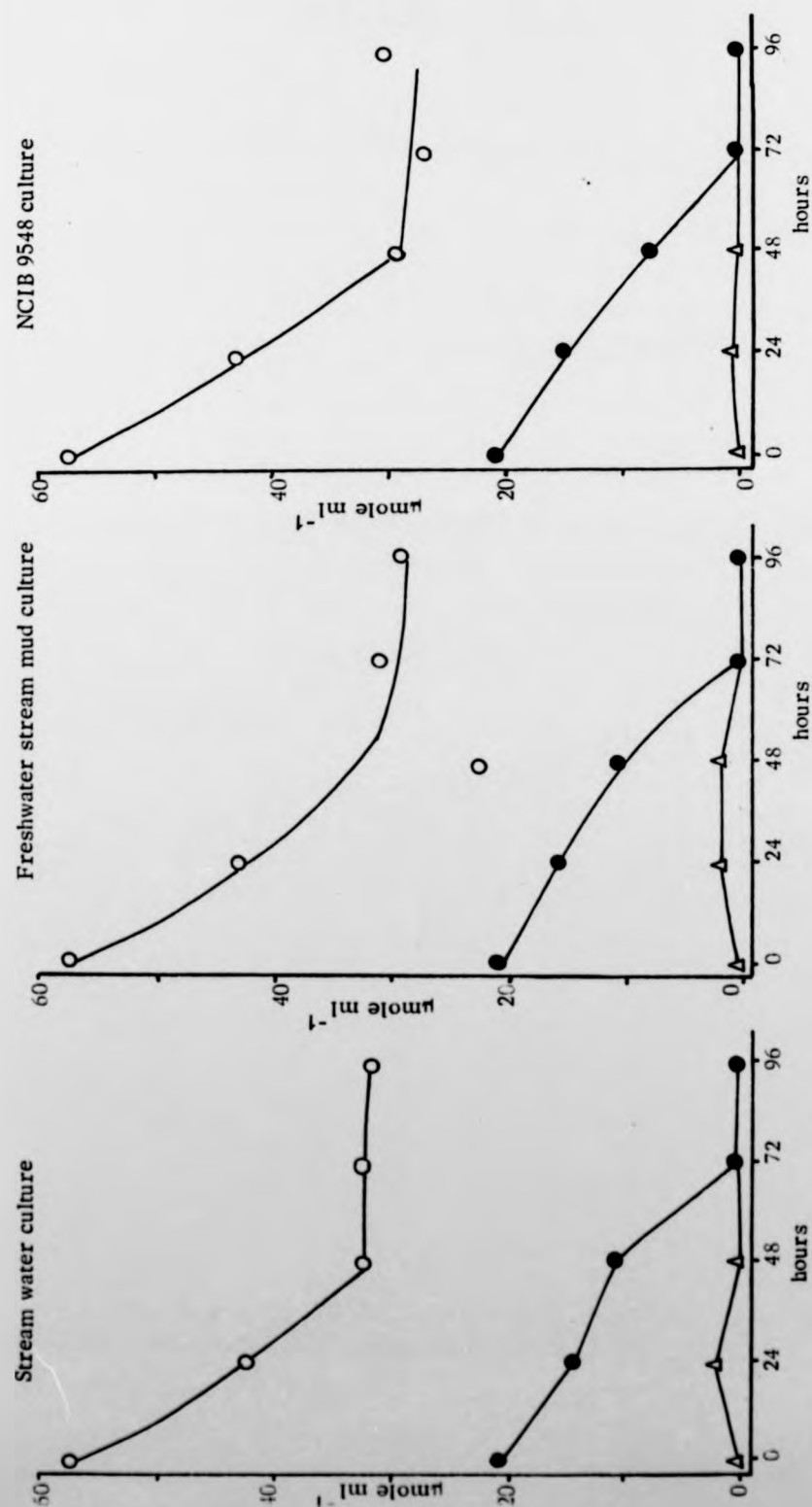


FIG. 9b Substrate consumption on medium ratio 5 thiosulphate : 16 nitrate. ●, S<sub>2</sub>O<sub>3</sub><sup>2-</sup>; Δ, S<sub>3</sub>O<sub>6</sub><sup>2-</sup>; ○, NO<sub>3</sub><sup>-</sup>.

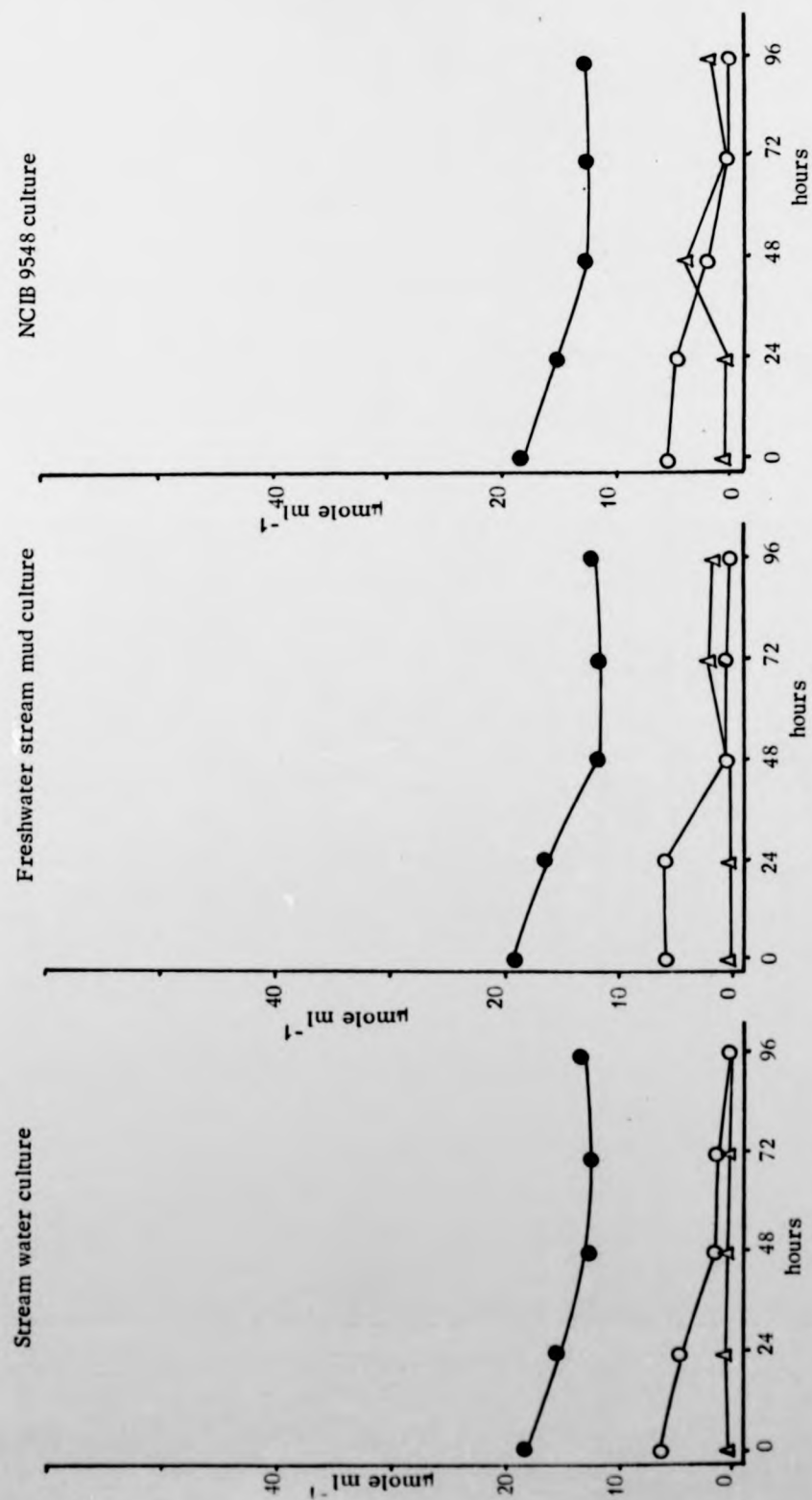


FIG. 9c Substrate consumption on medium ratio 5 thiosulphate : 2 nitrate. ●,  $\text{S}_2\text{O}_3^{2-}$ ; △,  $\text{S}_3\text{O}_6^{2-}$ ; ○,  $\text{NO}_3^-$ .

carbon dioxide being supplied from the atmosphere. For all future work bicarbonate was omitted from the aerobic medium.

A check was carried out on the aeration of the batch cultures. 250 ml conical flasks containing 100 ml medium were found to be only partially aerobic with little thiosulphate used and nitrite accumulating. With 25 ml media in these flasks, the thiosulphate was completely oxidized and no nitrite accumulated (Fig. 10a, b).

### 3.3 Chemostat Cultures

A continuous culture of *T. denitrificans* was established in a variety of nutrient limiting media which had been previously found suitable in batch cultures.

#### 3.3.1 Anaerobic nitrate-limited chemostat culture

Commencing with a late exponential phase, batch culture continually flushed with 5% carbon dioxide in nitrogen, a continuous culture was established and run without difficulty for 1861 h, using a medium containing 19.80 mM thiosulphate and 24.52 mM nitrate, the nitrate acting as the limiting nutrient (equation 31).

Steady states were maintained at seven dilution rates between 0.02 and  $0.08 \text{ h}^{-1}$ . Wash-out occurred at  $D = 0.09 \text{ h}^{-1}$ .

$$\text{Dilution rate} = \frac{\text{flow rate in ml/hour}}{\text{volume of culture}}$$

$$\text{Actual flow rate is when } \mu = \frac{\log_e 2}{\text{doubling time (hr)}} \quad \text{h}^{-1} = D$$

The precise determination of the input concentrations of thiosulphate and nitrate, as well as the steady state concentrations of these and of the biomass enabled the estimation of steady state yields in terms of g dry weight (or g protein) per mole thiosulphate consumed (Fig. 11). Residual nitrate was almost undetectable and was no more than 5 to 7% of the input concentration. Nitrite, sulphur and polythionates did not accumulate. About 3 mM thiosulphate remained at low D values, decreasing to 1 mM at  $D = 0.08 \text{ h}^{-1}$ . Biomass and yield increased with increased dilution rate (Fig. 11). The yield increased from 7.03 g dry



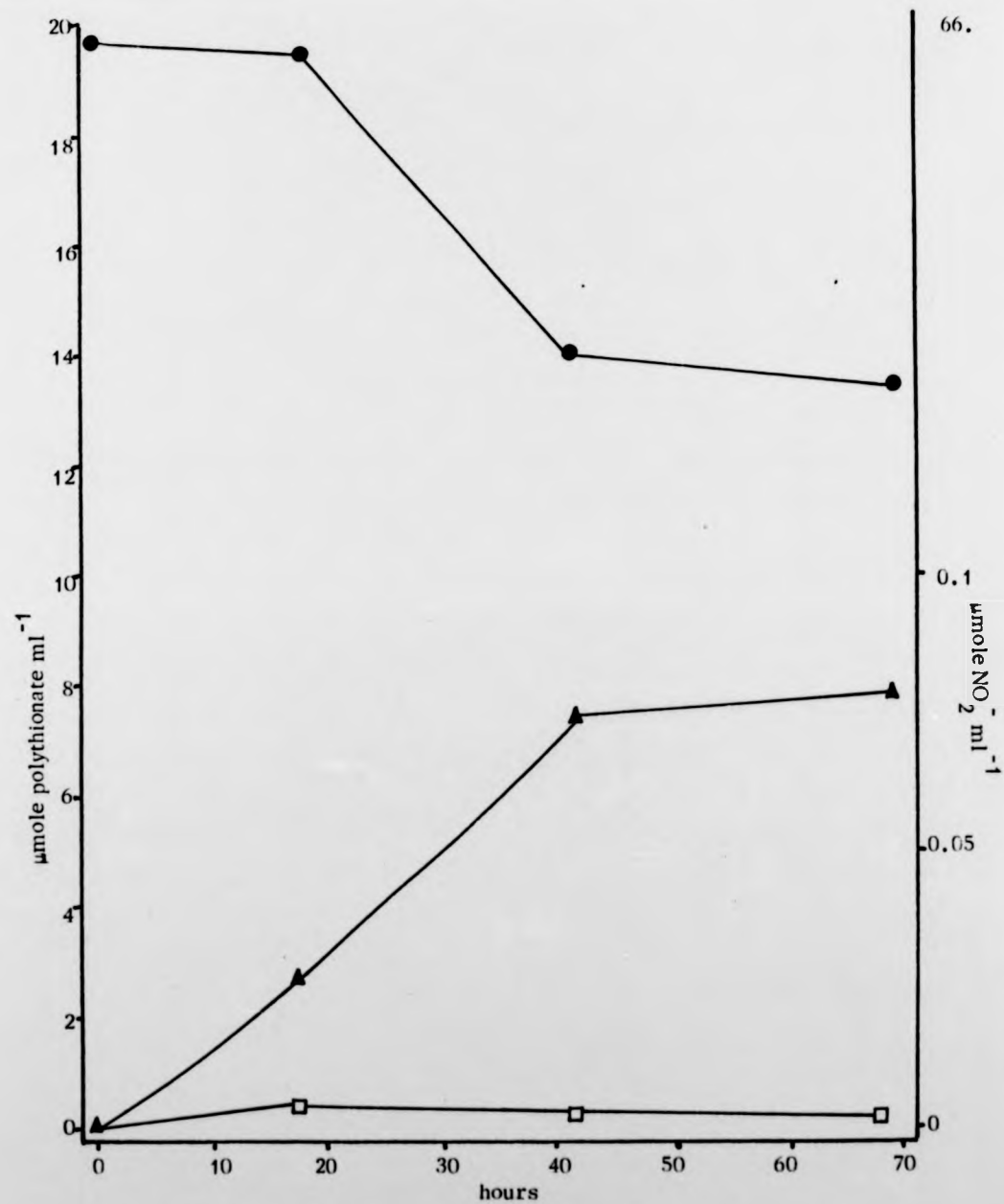


FIG. 10a Substrate consumption of *T. denitrificans* (NCIB 9548) in 250 ml flasks containing 100 ml medium (average of 3). ●,  $S_2O_3^{2-}$ ; □,  $S_4O_6^{2-}$ ; ▲,  $NO_2^-$ .

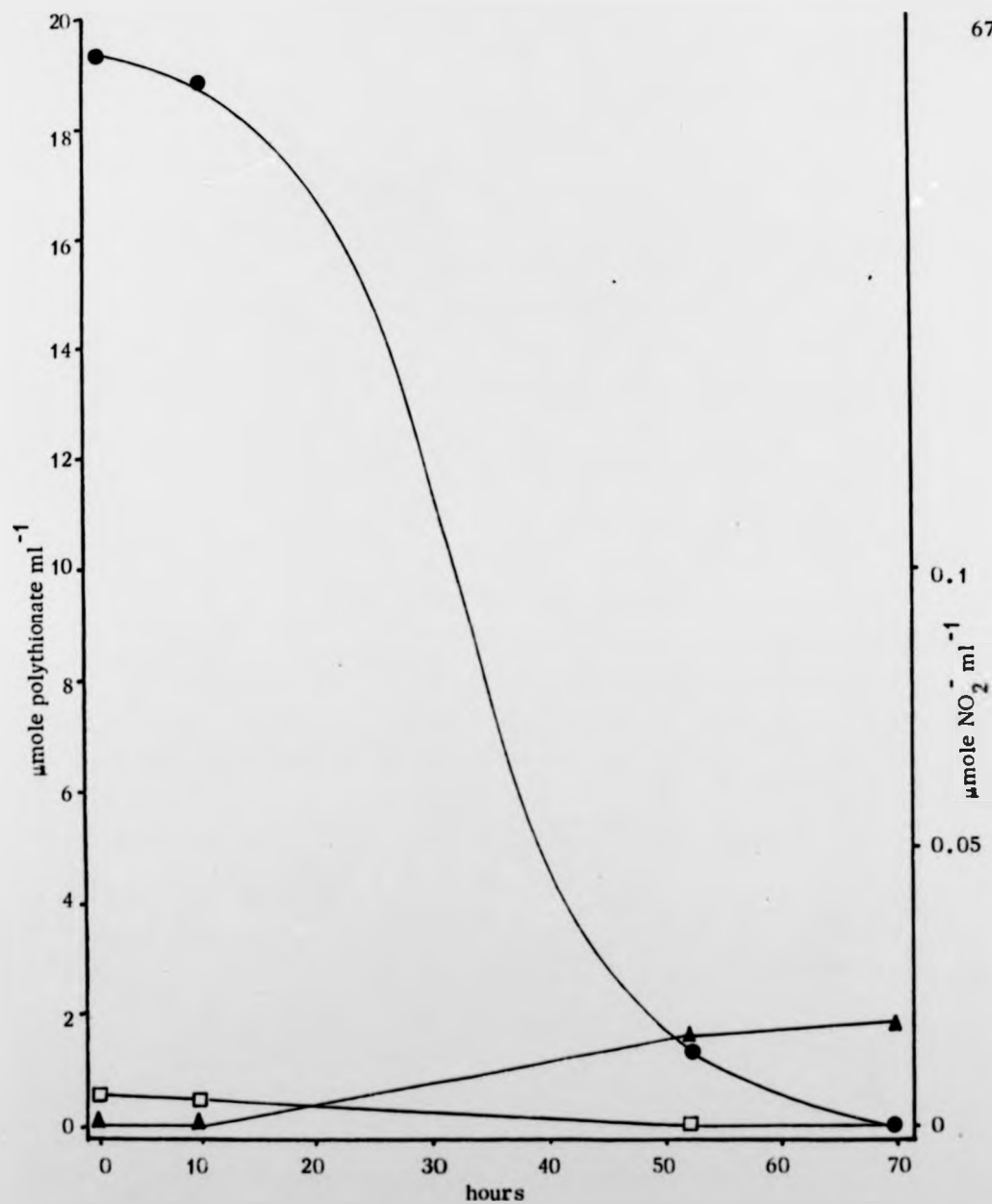


FIG. 10b Substrate consumption of *T. denitrificans* (NCIB 9548) in 250 ml flasks containing 25 ml medium. ●,  $\text{S}_2\text{O}_3^{2-}$ ; □,  $\text{S}_4\text{O}_6^{2-}$ ; ▲,  $\text{NO}_2^-$ .

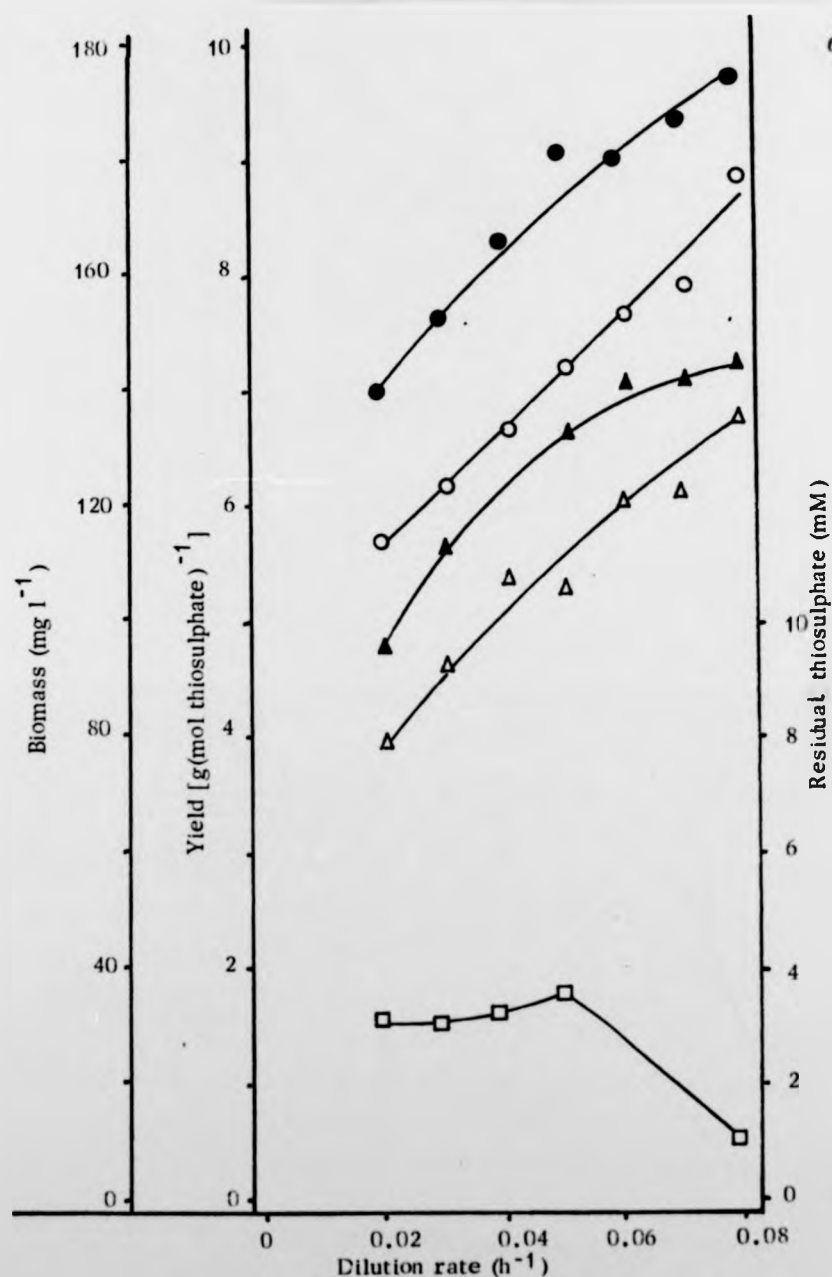


FIG. 11 Steady state biomass and yield of *T. denitrificans* grown in anaerobic chemostat culture on thiosulphate with nitrate limitation. Biomass (mg l<sup>-1</sup>) as dry wt. (○) and protein (Δ); yield [g(mol thiosulphate oxidized)<sup>-1</sup>] as dry wt. (●) and protein (Δ); unoxidized thiosulphate (□).

wt. per mol thiosulphate oxidized at  $D = 0.02 \text{ h}^{-1}$  to 9.7 at  $D = 0.08 \text{ h}^{-1}$ . The mean protein content calculated as percentage w/w of dry weight did not vary with dilution rate and was  $75.8 \pm 3.7\%$  (average of seven dilution rates). The reciprocal of the yields was plotted against reciprocal of  $D$ . This produced a linear graph (Fig. 12) from which the true growth yield ( $Y_g$ ) and apparent maintenance coefficient ( $m$ ) could be calculated (Pirt, 1965). In this case the  $Y_g$  was  $11.63 \text{ g dry wt. (mol thiosulphate)}^{-1}$  or  $8.51 \text{ g protein (mol thiosulphate)}^{-1}$  and  $m$  was  $1.4 \text{ mmol thiosulphate h}^{-1} (\text{g dry wt.})^{-1}$  (Table 4).

The specific rate of thiosulphate oxidation [ $q$  in  $\text{mmol h}^{-1} (\text{g dry wt.})^{-1}$ ] in the steady state chemostat cultures was calculated for each dilution rate from the thiosulphate consumption rate and steady state biomass. The value of  $q$  increased from 2.85 at  $D = 0.02 \text{ h}^{-1}$  to 8.24 at  $D = 0.08 \text{ h}^{-1}$  and gave a linear graph (fitted by regression analysis) when plotted against  $D$  (Fig. 13). This plot gave an alternative means of calculating  $Y_g$  and  $m$  where  $Y_g$  = the reciprocal of the slope of the  $q$  vs.  $D$  plot, as 11.1, and  $m$  = the  $q$  intercept of the  $q$  vs.  $D$  plot as 1.17. The mean values from the two procedures for determining  $Y_g$  and  $m$  were thus  $11.37 \text{ g dry wt. (mol thiosulphate)}^{-1}$  and  $1.29 \text{ mmol thiosulphate h}^{-1} (\text{g dry wt.})^{-1}$ .

A typical substrate and product balance for these steady states was given at  $D = 0.06 \text{ h}^{-1}$  at which the oxidation of 17.07 mM thiosulphate was accompanied by the disappearance of 22.75 mM nitrate and the production of 155 mg dry wt. bacteria giving a yield of 9.08 g (mol thiosulphate) $^{-1}$ . This corresponds to a nitrate:thiosulphate ratio of 1.33 which is lower than the theoretical ratio of 1.6 given by the oxidation equation (31). The production of 155 mg biomass is, however, equivalent to 73.5 mg C (6 mmol carbon dioxide) fixed, and consequently to the use of 3 mmol thiosulphate to provide reducing equivalents for fixation. Thus the thiosulphate consumed for energetic purposes, in this case nitrate reduction, was only  $17.07 - 3$ , i.e. 14.07 mmol, giving a true nitrate: thiosulphate ratio of 1.62 in accordance with the equation of thiosulphate oxidation.

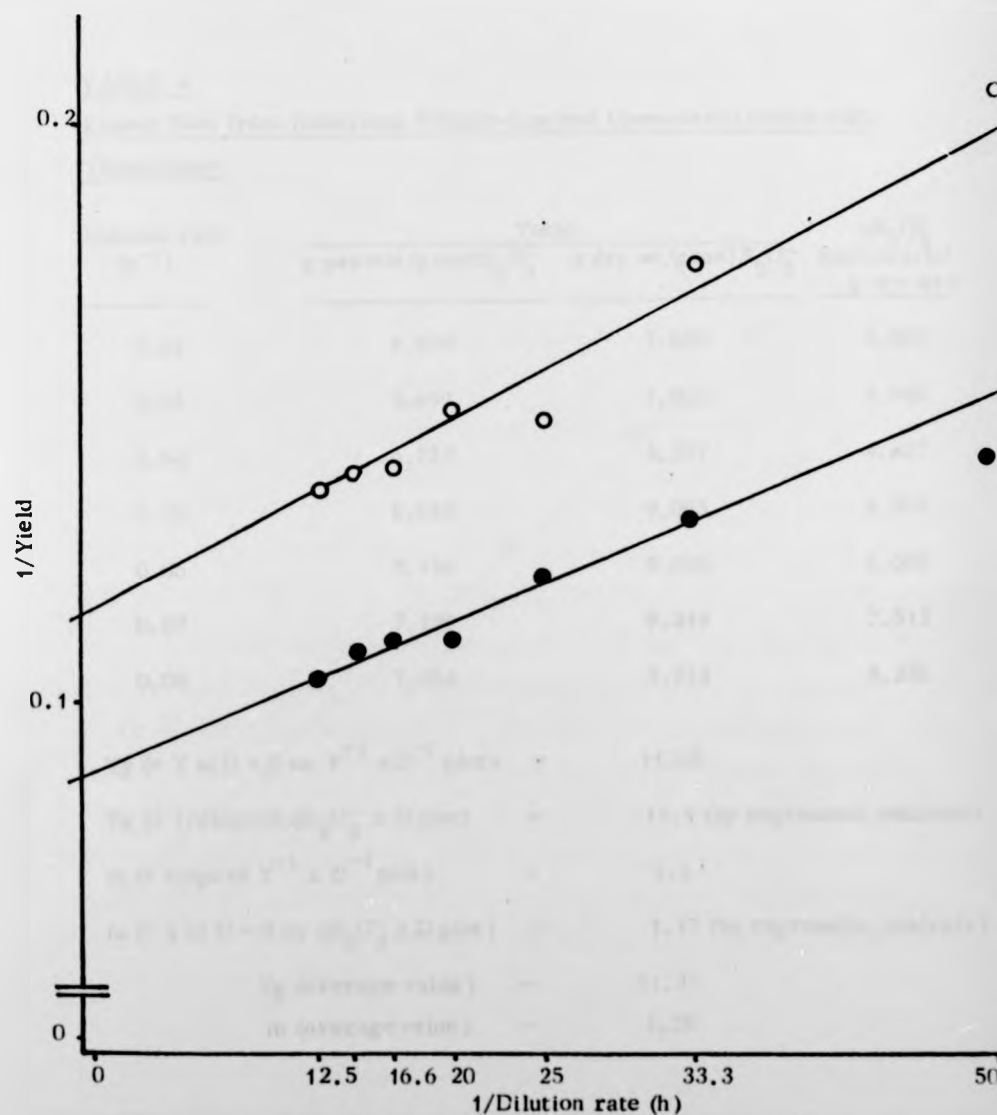


FIG. 12 Double reciprocal plots of dilution ratio and yields from anaerobic chemostat culture grown on thiosulphate with nitrate limitation;  $1/[g \text{ dry wt. (mol thiosulphate oxidized)}^{-1}]$  (●), and  $1/[g \text{ protein (mol thiosulphate oxidized)}^{-1}]$  (O).

TABLE 4

Kinetic Data from Anaerobic Nitrate-Limited Chemostat Culture with Thiosulphate

Dilution rate (h <sup>-1</sup> )	Yield		qS <sub>2</sub> O <sub>3</sub> <sup>2-</sup> (mmoles/h/ g dry wt)
	g protein/g mol S <sub>2</sub> O <sub>3</sub> <sup>2-</sup>	g dry wt/g mol S <sub>2</sub> O <sub>3</sub> <sup>2-</sup>	
0.02	4.879	7.025	2.850
0.03	5.699	7.608	3.946
0.04	6.737	8.287	4.827
0.05	6.688	9.063	5.519
0.06	7.136	9.080	6.609
0.07	7.179	9.319	7.512
0.08	7.354	9.714	8.236

$$Y_g (= Y \text{ at } D = 0 \text{ on } Y^{-1} \times D^{-1} \text{ plot}) = 11.63$$

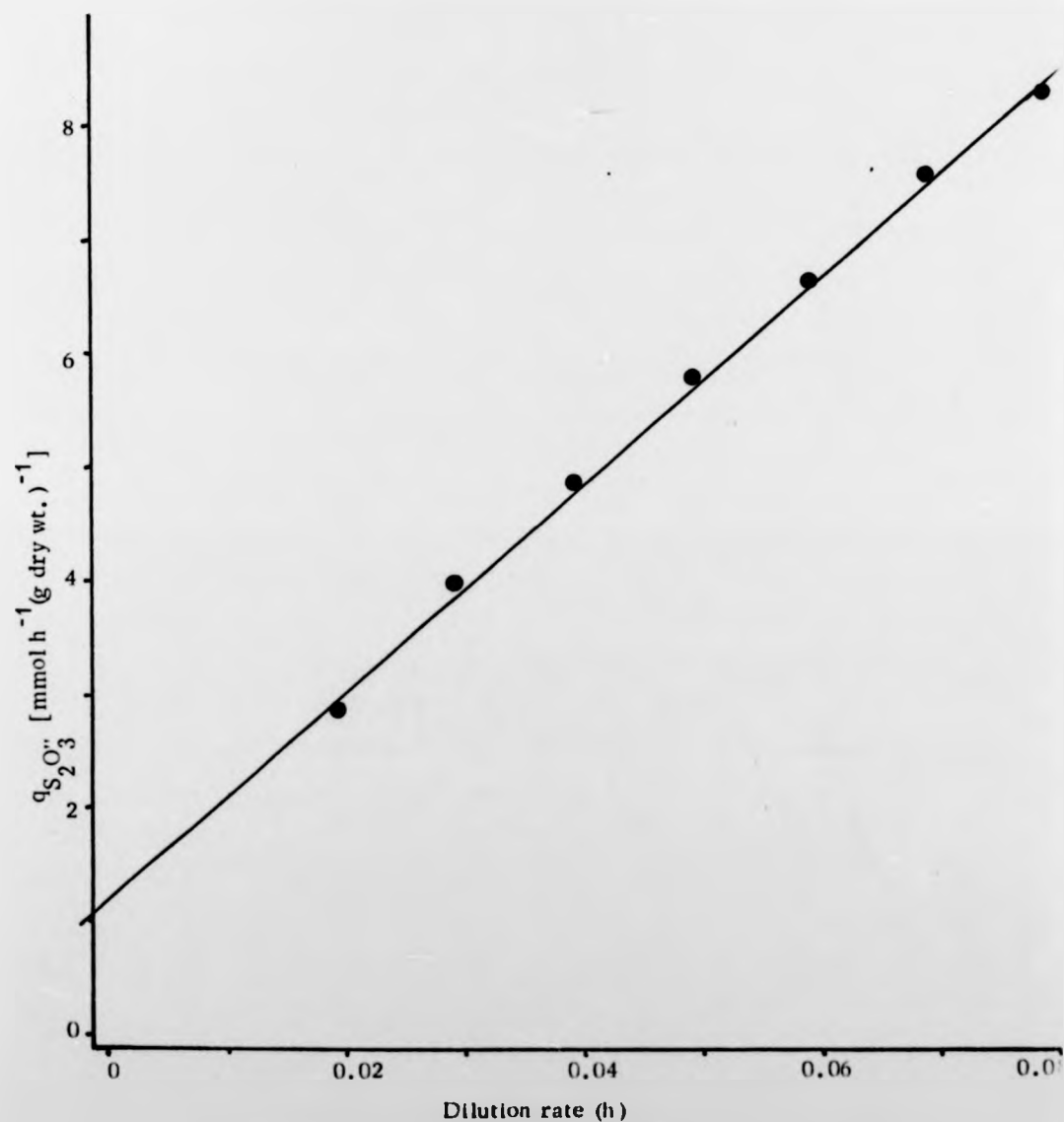
$$Y_g (= 1/\text{slope of } qS_2O_3^{2-} \times D \text{ plot}) = 11.1 \text{ (by regression analysis)}$$

$$m (= \text{slope of } Y^{-1} \times D^{-1} \text{ plot}) = 1.4$$

$$m (= q \text{ at } D = 0 \text{ on } qS_2O_3^{2-} \times D \text{ plot}) = 1.17 \text{ (by regression analysis)}$$

$$Y_g \text{ (average value)} = 11.37$$

$$m \text{ (average value)} = 1.29$$



**FIG. 13** Plot of specific rate of thiosulphate consumption against dilution rate of anaerobic chemostat culture grown on thiosulphate with nitrate limitation (by regression analysis).

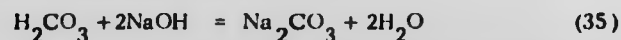
Attempts were carried out to relate the alkali uptake to maintain the pH with the amount of thiosulphate oxidized. At 0 hours, the total thiosulphate present was 14,850  $\mu$ mole, 12,525  $\mu$ mole nitrate and no nitrite. After 165 hours the total alkali added was 11,050  $\mu$ mole NaOH. From the basic equation of:



it is assumed that for every 5 thiosulphates, one sulphuric acid is produced. To balance the acid, two hydroxides would be needed. Therefore 11,050  $\mu$ mole NaOH is equivalent to 5,525  $\mu$ mole  $\text{H}_2\text{SO}_4$ . From the experimental data, 5940  $\mu$ mole  $\text{H}_2\text{SO}_4$  should be produced. After 165 hours, 187.5  $\mu$ mole nitrite has accumulated. According to the equation by Ishaque and Aleem (1973):



so 93.75  $\mu$ mole thiosulphate would be required to reduce 187.5  $\mu$ mole nitrite. So the amount of thiosulphate converted to acid is 14,856.25 equivalent to 5,902.5  $\mu$ mole  $\text{H}_2\text{SO}_4$  produced which is still more than found experimentally. All the way through there was a slight difference between the theoretical and experimental. Later the effect of 5%  $\text{CO}_2$  in nitrogen on a phosphate solution was measured. It was found that after 100 hours, 11,500  $\mu$ mole NaOH were added in the absence of bacterial activity (Fig. 14). This was presumably a consequence of acidification of the medium by  $\text{CO}_2$  as follows:



This led to the conclusion that no relation could be estimated between thiosulphate oxidation and alkali consumption.

Electron microscopic examination of this culture showed typical rod-shaped thionacilli (Fig. 15).

### 3.3.2 Anaerobic nitrate-limited chemostat culture growing on tetrathionate instead of thiosulphate

20 mM thiosulphate was replaced by 11.4 mM tetrathionate with approximately 20 mM nitrate under the same conditions employed in the previous section.





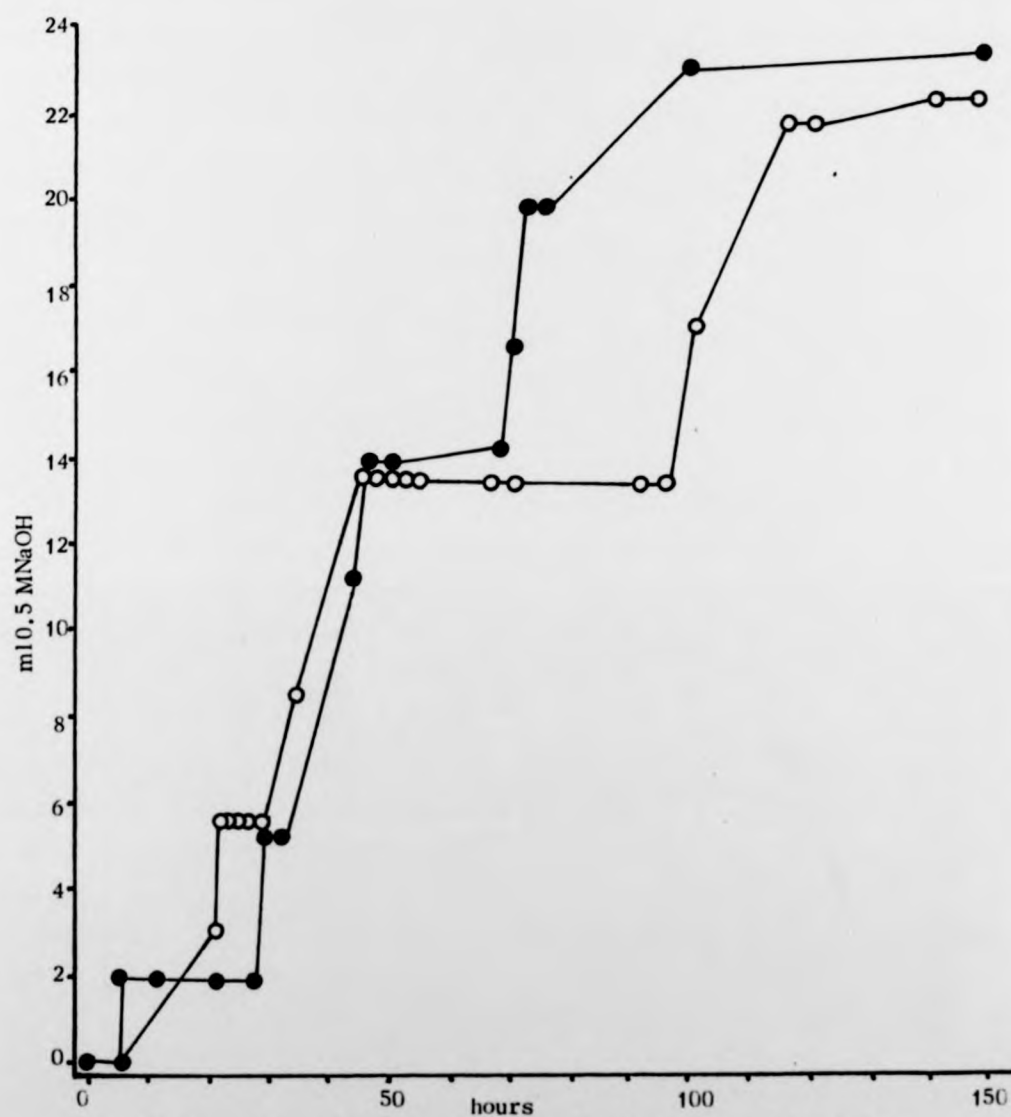
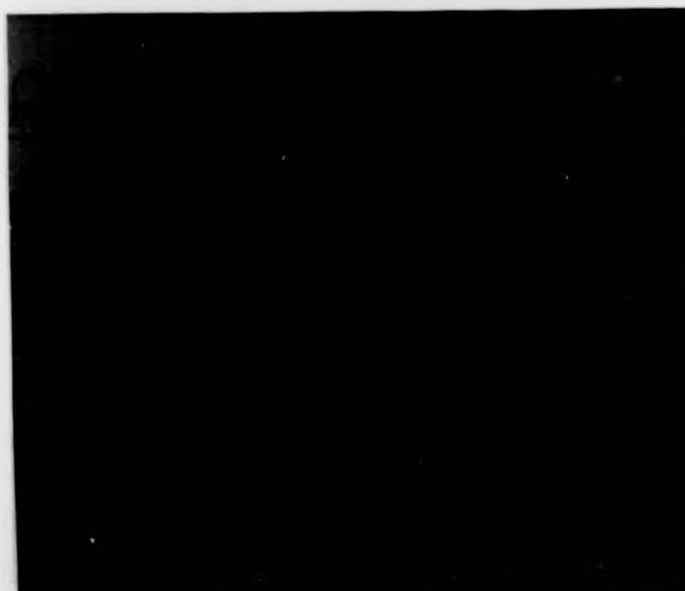


FIG. 14 Comparison of pH control between *T. denitrificans* (NCIB 9548) culture (○) and  $\text{KH}_2\text{PO}_4$  solution (●).

FIG. 15 Electron microscope photographs of *T. denitrificans*

(a)



(b)



(a) *T. denitrificans* (NCIB 9548) in continuous culture

(b) *T. denitrificans* isolated from freshwater stream (batch culture)

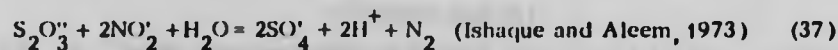
Stain = 1% <sup>(W/V)</sup> uranyl acetate; magnification = x 20,000

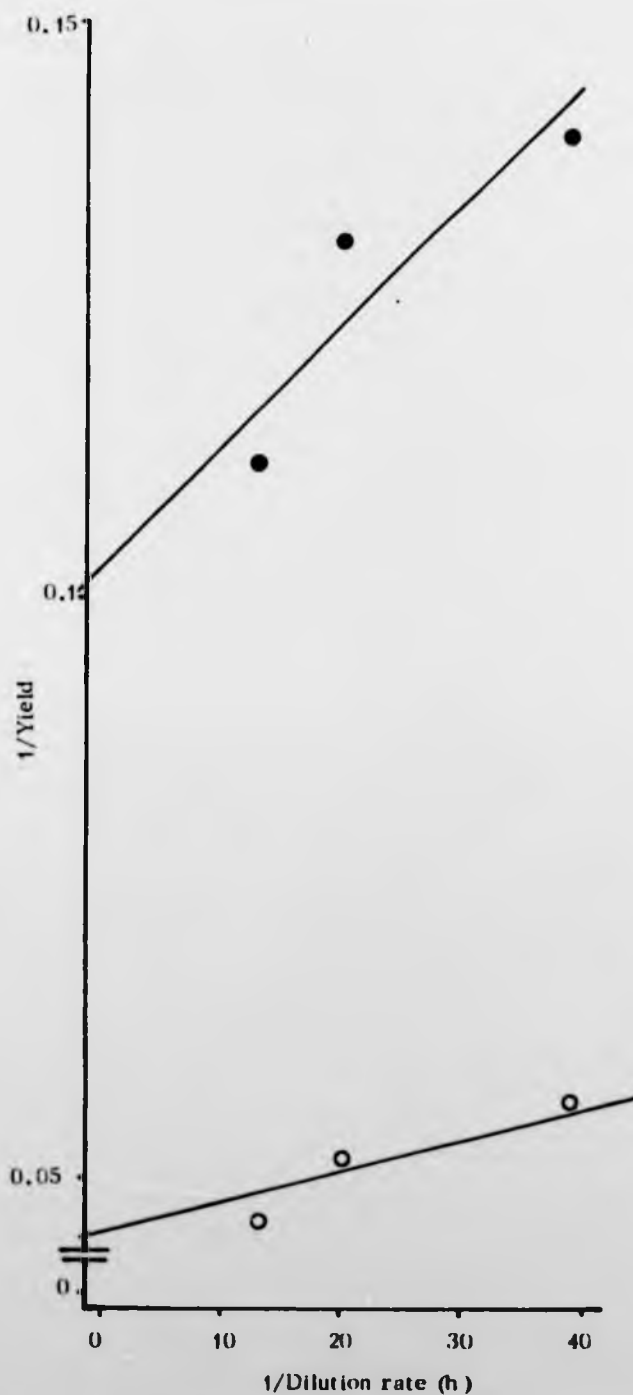
Steady states were maintained at three dilution rates of 0.025, 0.05, 0.076 h<sup>-1</sup> for a total period of 1299 h. Wash-out occurred at D = 0.08 h<sup>-1</sup>. More than 98% of the tetrathionate was consumed with small amounts of thiosulphate (0.07 - 0.13 mM) and trithionate (0.15 - 0.26 mM) being produced. Not all the nitrate was reduced to nitrogen, as 0.025 mM nitrite was found present and 1.11 mM nitrate remained. The yield figures for D = 0.025, 0.05, 0.076 h<sup>-1</sup> were respectively 18.10 and 20.99 g dry wt. (mol tetrathionate)<sup>-1</sup> indicating a Y<sub>g</sub> of 21.34 g dry wt. (mol tetrathionate)<sup>-1</sup> calculated as an average from the qS<sub>4</sub>O<sub>6</sub><sup>2-</sup> vs. D plot and 1/Y vs. 1/D plot (Fig. 16, Table 5a) which is as expected due to the double number of sulphur atoms in tetrathionate.

### 3.3.3 Anaerobic nitrite-limited chemostat culture

The use of the chemostat with a limiting oxidant enabled the use of normally toxic materials such as nitrite (Baalsrud and Baalsrud, 1954). The presence of nitrite with thiosulphate resulted in the thiosulphate being all converted to tetrathionate in the absence of any bacteria. This was overcome by pumping the thiosulphate and nitrite into the media from different sources. The nitrite limited culture was switched from a supply of 20 mM thiosulphate with 20 mM nitrate to a supply of 21 mM thiosulphate and 20 mM nitrite. Two steady states were sustained at dilution rates of 0.07 and 0.08 h<sup>-1</sup> over a period of 469 h, with wash-out occurring at 0.08 h<sup>-1</sup>. Nitrite was completely consumed but small amounts of trithionate (0.87 mM) were detectable and negligible amounts of tetrathionate. 7.51 mM thiosulphate was left unused, therefore 13.49 mM was consumed. The steady state biomass at D = 0.07 h<sup>-1</sup> was 149 mg dry wt. l<sup>-1</sup> indicating a yield of 11.04 g (mol thiosulphate)<sup>-1</sup> (Table 5b).

The nitrite : thiosulphate ratio from these results is 1.48 which is lower than the theoretical ratio of 2.





**FIG. 16** Double reciprocal plots of dilution rates and yields from anaerobic chemostat culture grown on tetrathionate with nitrate limitation (by regression analysis):  $1/[\text{g dry wt. (mol tetrathionate oxidized)}^{-1}]$  (○), and  $1/[\text{g protein (mol tetrathionate oxidized)}^{-1}]$  (●).

TABLE 5

(a) Kinetic Data from Anaerobic Nitrate Limited Chemostat Data with Tetrathionate

Dilution rate (h <sup>-1</sup> )	Yield		qS <sub>4</sub> O <sub>6</sub> (mmole/h/g dry wt)
	g protein/g mol S <sub>4</sub> O <sub>6</sub> <sup>2-</sup>	g dry wt/g mol S <sub>4</sub> O <sub>6</sub> <sup>2-</sup>	
0.025	7.07	18.10	1.249
0.05	7.63	19.68	2.441
0.076	8.98	20.99	3.702

$$Y_g (= Y \text{ at } D = 0 \text{ on } Y^{-1} \times D^{-1} \text{ plot}) = 22.22 \text{ (by regression analysis)}$$

$$Y_g (= 1/\text{slope of } qS_4O_6 \times D \text{ plot}) = 20.46 \text{ (by regression analysis)}$$

$$m (= \text{slope of } Y^{-1} \times D^{-1} \text{ plot}) = 0.0876 \text{ (by regression analysis)}$$

$$m (= q \text{ at } D = 0 \text{ on } qS_4O_6 \times D \text{ plot}) = 0.0938 \text{ (by regression analysis)}$$

$$Y_g (\text{average}) = 21.34$$

$$m (\text{average}) = 0.0907$$

(b) Kinetic Data from Anaerobic Nitrite Limited Chemostat Data with Thiosulphate

Dilution rate (h <sup>-1</sup> )	Yield	
	g protein/g mol S <sub>2</sub> O <sub>3</sub> <sup>2-</sup>	g dry wt/g mol S <sub>2</sub> O <sub>3</sub> <sup>2-</sup>
0.07	5.63	11.04

The production of 70.6 mg (5.88 carbon dioxide fixed) requires 2.94 mmol thiosulphate to provide the necessary reducing equivalents. So in this case, the thiosulphate consumed for energetic purposes is  $13.49 - 2.94$ , i.e. 10.55 mM which gives a true nitrite:thiosulphate ratio of 1.89. The small amount of trithionate produced may account for the ratio not being exactly 2.

#### 3.3.4 Anaerobic thiosulphate-limited chemostat culture

A thiosulphate-limited culture was initiated from a nitrate limited culture at  $D = 0.02 \text{ h}^{-1}$  which led to the immediate uptake of all the thiosulphate involving no lag period. With the thiosulphate (17 mM) as the limiting nutrient and nitrate supplied in excess at 30 mM, virtually all the thiosulphate was completely oxidized with no polythionates or sulphur forming throughout the run. Eight steady states were maintained from  $D = 0.021 - 0.083 \text{ h}^{-1}$  over a period of 2018 h, with wash-out occurring at  $0.09 \text{ h}^{-1}$ .

Between the dilution rates of  $0.04 \text{ h}^{-1}$  to  $0.083 \text{ h}^{-1}$  the nitrate consumption frequently exceeded the theoretical requirement (Table 6). This coincided with nitrite formation and disappearance, which showed harmonic oscillations at fixed dilution rates with little dampening at high D values (Fig. 17). The effluent gases from the culture contained small amounts of nitrous oxide and nitric oxide (detected as nitrogen dioxide). Nitrogen dioxide is not believed to be an intermediate in the nitrate reduction pathway (Aminuddin and Nicholas, 1973; Ishaque and Aleem, 1973; Baldensperger and Garcia, 1975).

Biomass and yield tended to increase at high D values in a similar manner to the nitrate limited cultures (Table 7) as was previously shown at a single dilution rate ( $0.03 \text{ h}^{-1}$ ) by Timmer-ten Hoor (1976). A greater variability in the yield (and apparent protein content of cultures) was found with thiosulphate limited cultures than with those limited by nitrate. This may be due to the variable nitrite accumulation (Cheah, 1973) (Table 6).

TABLE 6

Comparison of Nitrite Accumulation, Nitrate remaining and Protein  
Content in Anaerobic Thiosulphate-limited Cultures of  
*T. denitrificans*

Dilution rate ( $\text{h}^{-1}$ )	Hours	mM $\text{NO}_2^-$	mM $\text{NO}_3^-$	mg protein
0.04	749.05	0.048	4.75	61.25
	869.56	1.380	2.47	46.25
	893.22	0.115	3.36	50.00
	909.22	1.535	2.57	46.25
0.05	1012.26	1.910	2.77	63.75
	1077.23	0	3.46	64.38
0.06	1414.31	0	2.18	82.19
	1486.78	1.36	0.79	76.88
	1581.07	0.79	1.58	93.13

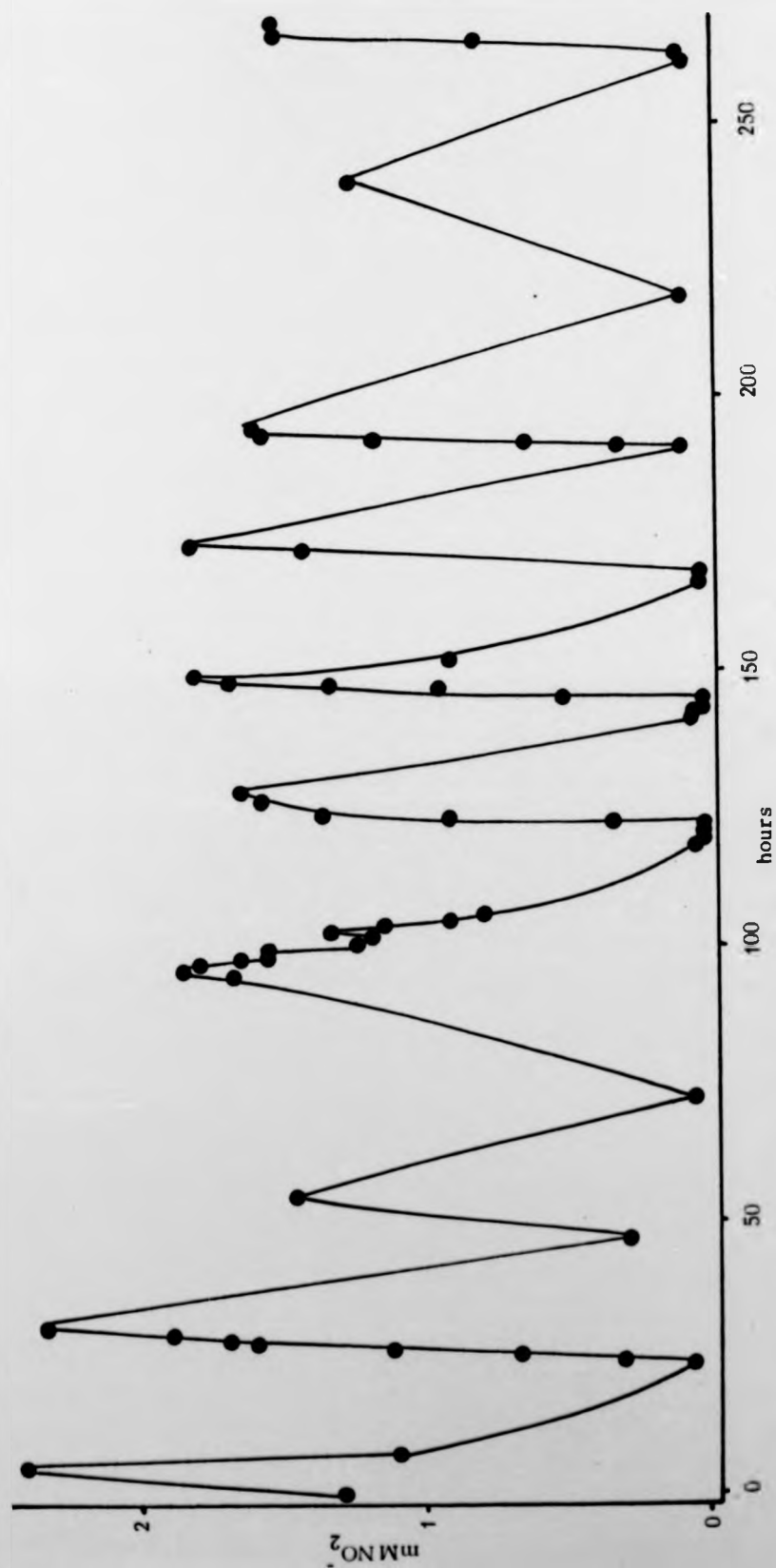


FIG. 17 Nitrite oscillations in thiosulphate-limited anaerobic culture ( $D = 0.05 \text{ h}^{-1}$ )



TABLE 7 Comparison of steady state biomass, yield and thiosulphate consumption by T. denitrificans in (A) nitrate-limited anaerobic, (B) thiosulphate-limited anaerobic

Input thiosulphate concentrations were 20 mM for A and 17 mM for B

Dilution rate (h <sup>-1</sup> )*	Steady state biomass (mg dry wt l <sup>-1</sup> )		Steady state thiosulphate consumption (mmol l <sup>-1</sup> )		Yield (g protein/g mol thiosulphate)		Yield (g dry wt/g mol thiosulphate)	
	A	B†	A	B†	A	B†	A	B†
0.02	115	119	16.4	15.4	4.88	4.12	7.03	6.88
0.03	125	117	16.4	17.0	5.70	3.96	7.61	5.59
0.04	135	100	16.3	17.0	6.74	3.35	8.29	6.77
0.05	145	123	16.0	17.0	6.69	4.16	9.06	7.94
0.06	155	142	17.1	17.0	7.14	4.81	9.08	8.24
0.07	160	147	17.2	17.0	7.18	4.98	9.32	9.12
0.08	180	132	18.5	17.0	7.35	4.47	9.71	10.29

† Mean values from more than one steady state determination

\* Approximate dilution rates given for ease of comparison: actual values were up to 5% greater than indicated

Due to this the determination of  $Y_g$  was somewhat imprecise and values between 9.2 and 14.2 g dry wt. (mol thiosulphate)<sup>-1</sup> could be deduced from the data from the  $qS_2O_3$  vs. D plot (Fig. 18) and 10.64 from the  $1/Y$  vs.  $1/D$  plot (Fig. 19). The first of the  $Y_g$  values from the  $q$  vs. D plot was based on the biomass being calculated from the protein figures, and the latter where the biomass was calculated from the optical density readings (Table 8). The mean value was 11.35 g dry wt. (mol thiosulphate)<sup>-1</sup> which is close to the anaerobic nitrate limited  $Y_g$  value of 11.37 (Table 1).

### 3.3.5 Anaerobic thiosulphate-limited chemostat culture with nitrous oxide replacing nitrate

An anaerobic thiosulphate-limited culture grown on nitrous oxide was initiated from an anaerobic thiosulphate-limited culture grown with nitrate. A transition phase of 50.25 h with a mixture of nitrous oxide and nitrate at a dilution rate of  $0.07 \text{ h}^{-1}$  caused the culture to wash out and nitrite to accumulate (1.73 mM). The culture was switched to a nitrate free media with nitrous oxide and to a dilution rate of  $0.04 \text{ h}^{-1}$ . The culture recovered, all the nitrite was consumed, a negligible amount of S appeared transitionally, and a steady state was established, giving a biomass of  $215 \text{ mg l}^{-1}$  as compared with  $135 \text{ mg l}^{-1}$  for anaerobic thiosulphate-limited with nitrate. Two steady states were established at  $0.037$  and  $0.04 \text{ h}^{-1}$  over a period of 463 h with complete thiosulphate consumption and no polythionates accumulating. Wash-out occurred between  $0.05$  and  $0.07 \text{ h}^{-1}$ . The wash-out process could not be stopped by lowering the dilution rate to the previous  $0.04 \text{ h}^{-1}$  or lower. The culture was saved by switching to "batch growth" and adding large amounts of thiosulphate. On switching to thiosulphate (20 mM) and nitrate (36 mM) media at a dilution rate of  $0.037 \text{ h}^{-1}$ , it immediately recovered, consuming all the thiosulphate with 1.45 mM nitrate remaining. The immediate switch back to nitrous oxide at the same dilution rate caused an increase in biomass from 135 mg to 200 mg and an intermediate accumulation of 0.83 mM nitrite. The yield at  $D = 0.037 \text{ h}^{-1}$  was  $10 \text{ g dry wt. (g mol S}_2\text{O}_3^{2-})^{-1}$ .

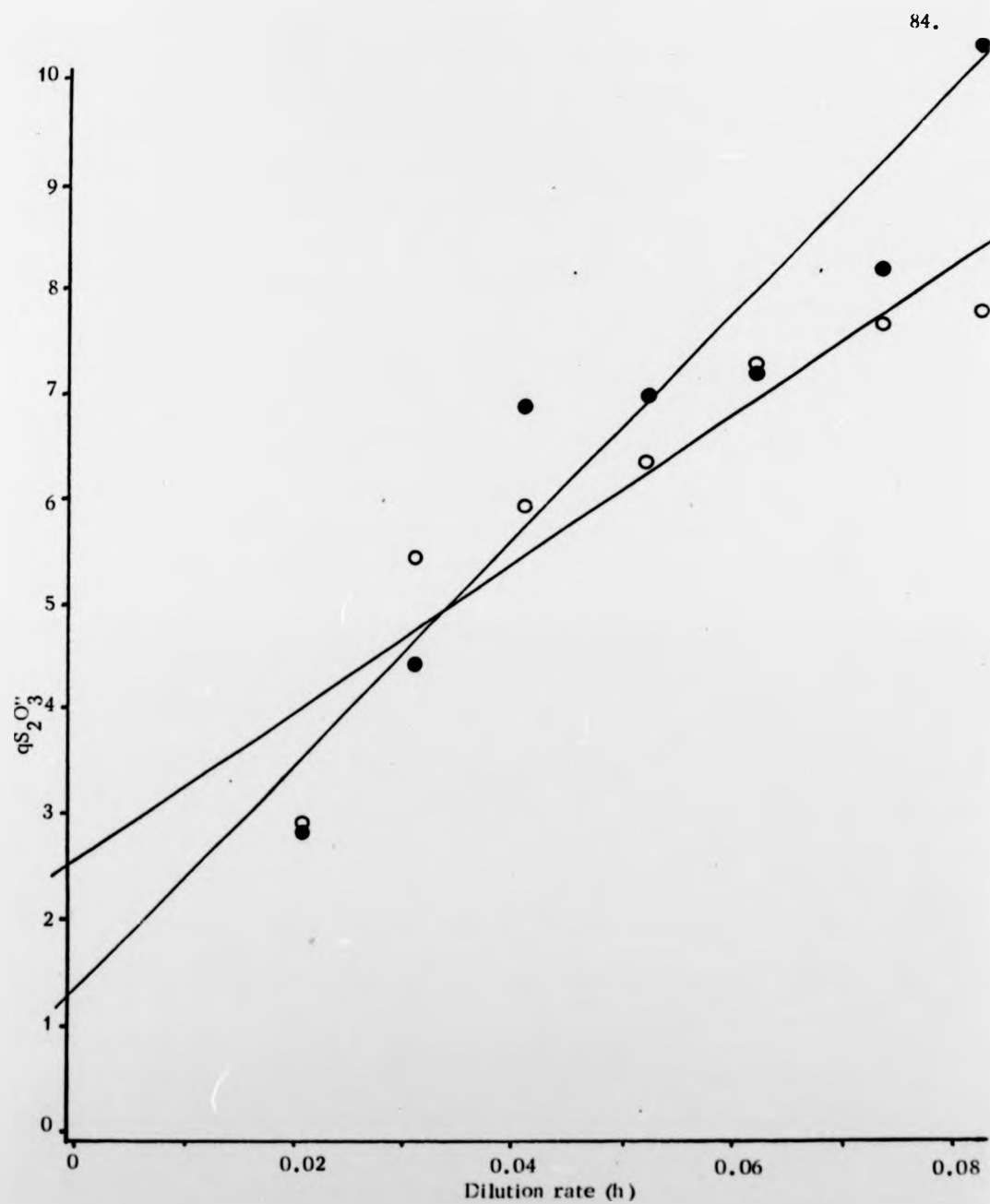
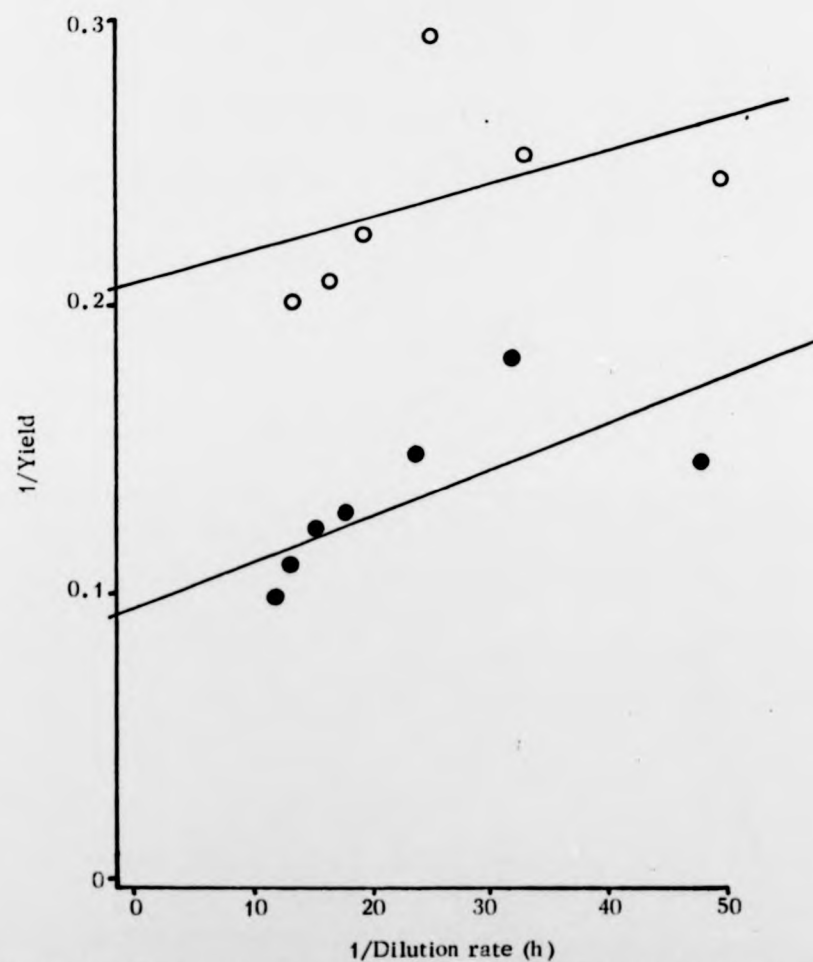


FIG. 18 Plot of specific rate of thiosulphate consumption against dilution rate of anaerobic chemostat grown culture with thiosulphate limitation (by regression analysis): (O)  $\text{mmol h}^{-1} (\text{g dry wt.})^{-1}$ ; (●)  $\text{mmol h}^{-1} (\text{g protein})^{-1}$ .



**FIG. 19** Double reciprocal plots of dilution rates and yields from anaerobic chemostat grown culture with thiosulphate limitation (by regression analysis):  $1/[g \text{ dry wt. (mol thiosulphate oxidized)}^{-1}]$  (●),  $1/[g \text{ protein (mol thiosulphate oxidized)}^{-1}]$  (○).

TABLE 8

Kinetic Data from Anaerobic Thiosulphate-Limited Chemostat Culture

Dilution rate (h <sup>-1</sup> )	Yield		qS <sub>2</sub> O <sub>3</sub> (mmole/h/g dry wt)
	g protein/g mol S <sub>2</sub> O <sub>3</sub> <sup>2-</sup>	g dry wt/g mol S <sub>2</sub> O <sub>3</sub> <sup>2-</sup>	
0.0208	4.124	6.876	2.913
0.0312	3.961	5.588	5.379
0.0413	3.399	6.765	5.913
0.0519	4.168	7.941	6.304
0.0619	4.814	8.235	7.286
0.0732	4.981	9.118	7.684
0.0831	4.475	10.294	7.771

$$Y_g (=Y \text{ at } D = 0 \text{ on } Y^{-1} \times D^{-1} \text{ plot}) = 10.64 \text{ (by regression analysis)}$$

$$Y_g (=1/\text{slope of } qS_2O_3 \times D \text{ plot}) = 14.2 \text{ (by regression analysis)}$$

$$m (= \text{slope of } Y^{-1} \times D^{-1} \text{ plot}) = 1.63 \text{ (by regression analysis)}$$

$$m (= q \text{ at } D = 0 \text{ on } qS_2O_3 \times D \text{ plot}) = 2.5 \text{ (by regression analysis)}$$

$$Y_g (\text{average}) = 12.42$$

$$m (\text{average}) = 2.07$$

with biomass calculated from dry weights

If biomass is calculated from protein figures,

$$\text{then } Y_g (=1/\text{slope of } qS_2O_3 \times D \text{ plot}) = 9.22 \text{ (by regression analysis)}$$

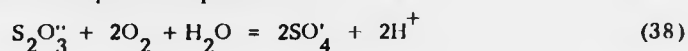
$$m (= q \text{ at } D = 0 \text{ on } qS_2O_3 \times D \text{ plot}) = 1.20$$

$$\therefore Y_g (\text{average}) = 11.35$$

$$m (\text{average}) = 1.78$$

### 3.3.6 Aerobic thiosulphate-limited chemostat culture

The aerobic chemostat culture was started from a late exponential phase batch culture flushed with air. No polythionates were formed as was previously recorded in batch cultures. The stoichiometry for aerobic thiosulphate respiration is:



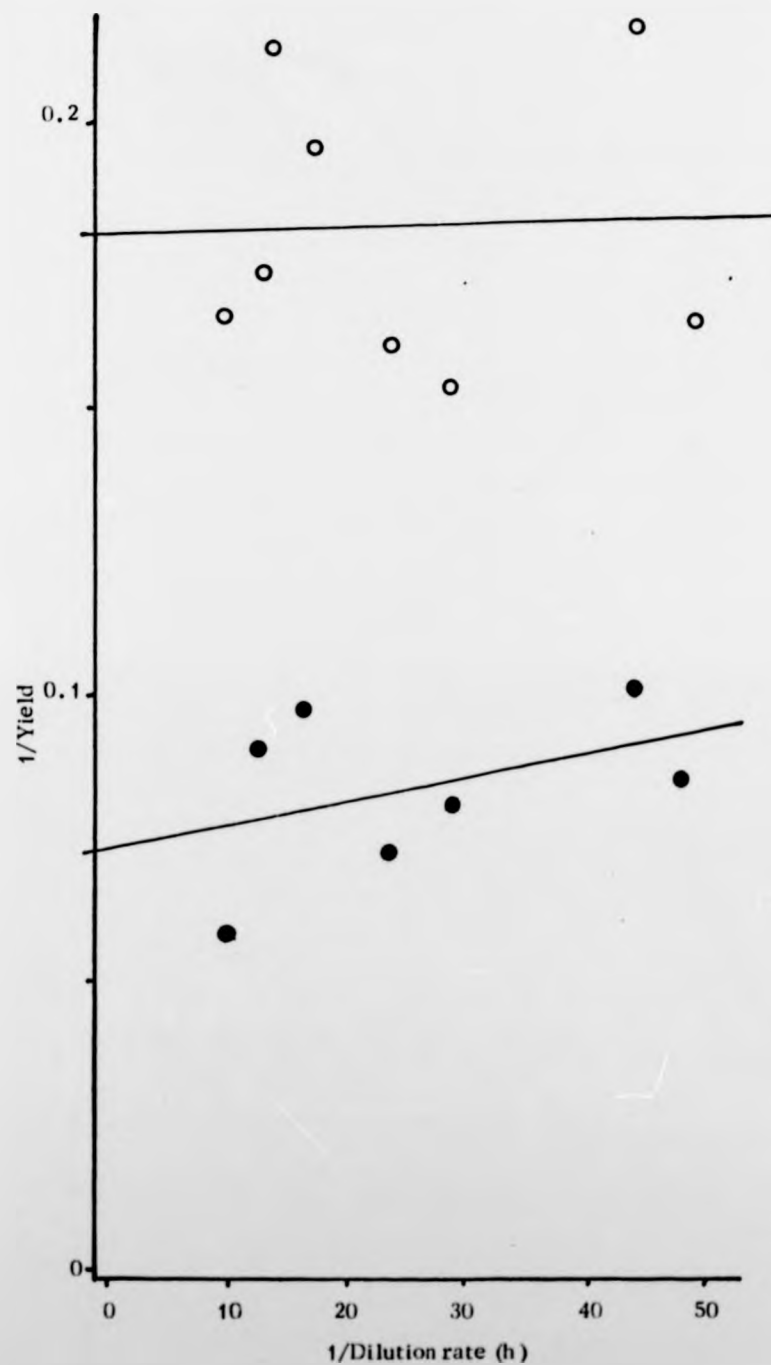
The thiosulphate was completely consumed in all steady states and was the growth-limiting substrate.

In two chemostat runs (2020 and 1300 h duration), eight steady states were established between  $D = 0.02$  to  $0.13 \text{ h}^{-1}$  with culture wash-out between  $0.13$  and  $0.14 \text{ h}^{-1}$ . At first the culture was aerated with air only at  $250 \text{ ml (1 litre culture volume)}^{-1} \text{ min}^{-1}$  and was found to be carbon dioxide limiting as the yield at  $D = 0.02 \text{ h}^{-1}$  was increased from  $7.5 \text{ g dry wt. (mol thiosulphate)}^{-1}$  with air to  $11.8$  when the gas flow was supplemented with  $5\% \text{ (v/v)}$  carbon dioxide. A similar phenomenon was seen with *T. ferrooxidans* grown on tetrathionate (Eccleston and Kelly, 1978) and presumably indicates dependence on carbon dioxide of the efficiency of energy coupling during thiosulphate oxidation. All subsequent steady states were attained with excess carbon dioxide supply and showed an increase in steady state yield with increasing dilution rate from  $10.7 \text{ g dry wt. (mol thiosulphate)}^{-1}$  at  $D = 0.02$  to  $12.8$  at  $D = 0.08 \text{ h}^{-1}$ .

$Y_g$  and  $m$  were calculated in two ways as before: (i)  $Y^{-1} \times D^{-1}$  plot (Fig. 20), (ii)  $q \times D$  plot (Fig. 21), to give an average  $Y_g$  of  $14.43 \text{ g dry wt. (mol thiosulphate)}^{-1}$  and  $m$  of  $0.56 \text{ mmol thiosulphate h}^{-1} \text{ (g dry wt.)}^{-1}$  (Table 9). This  $Y_g$  value is significantly greater than that of the anaerobic thiosulphate limited culture [ $11.35 \text{ g dry wt. (mol thiosulphate)}^{-1}$ ] (Table 10). This was expected, as more energy is available from the aerobic oxidation of thiosulphate than from its anaerobic oxidation (Timmer-ten Hoor, 1976; Kelly, 1978).

### 3.3.7 Specific rates of thiosulphate consumption

As described  $q_{\text{S}_2\text{O}_3^{2-}}$  and  $q_{\text{S}_4\text{O}_6^{2-}}$  were used as an alternative means of calculating  $Y_g$  and  $m$ . Values of  $q_{\text{S}_2\text{O}_3^{2-}}$  increased with  $D$  for all three



**FIG. 20** Double reciprocal plots of dilution rates and yields from aerobic chemostat grown culture with thiosulphate limitation (by regression analysis):  $1/[\text{g dry wt. (mol thiosulphate oxidized)}^{-1}]$  (●),  $1/[\text{g protein (mol thiosulphate oxidized)}^{-1}]$  (○).

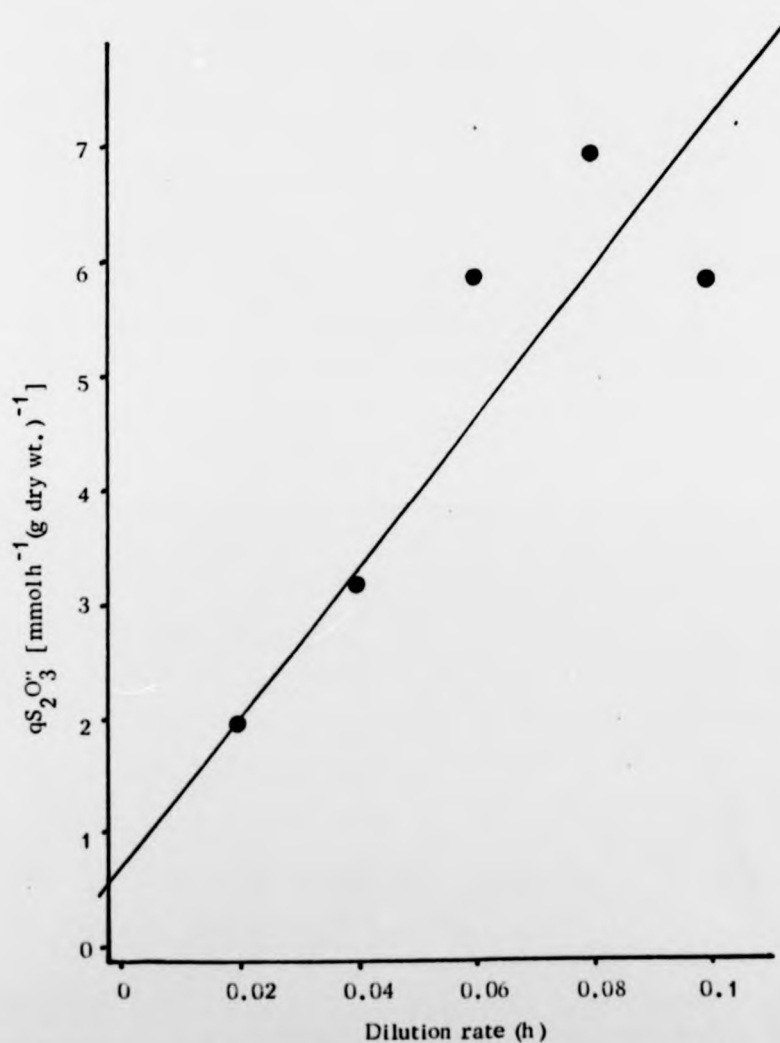


FIG. 21 Plot of specific rate of thiosulphate consumption against dilution rate of aerobic chemostat culture grown on thiosulphate (by regression analysis).



TABLE 9

Kinetic Data from Aerobic Thiosulphate-Limited Chemostat Culture

Dilution rate (h <sup>-1</sup> )	Yield		qS <sub>2</sub> O <sub>3</sub> <sup>2-</sup> (mmole/h/dry wt.)
	g protein/g mol S <sub>2</sub> O <sub>3</sub> <sup>2-</sup>	g dry wt./g mol S <sub>2</sub> O <sub>3</sub> <sup>2-</sup>	
0.0209	6.04	11.795	1.696
0.0225*	4.612	9.84	2.285
0.0419	6.55	12.308	3.250
0.0426*	6.157	13.889	3.067
0.0619	5.13	10.256	5.850
0.0808	4.72	12.821	6.240
0.0845*	5.74	11.111	7.605
0.1008	6.04	17.436	5.735

\* Results from a separate run

Y<sub>g</sub> (= Y at D = 0 on Y<sup>-1</sup> x D<sup>-1</sup> plot) = 13.70 (by regression analysis)Y<sub>g</sub> (= 1/slope of qS<sub>2</sub>O<sub>3</sub><sup>2-</sup> x D plot) = 15.15 (by regression analysis)m (= slope of Y<sup>-1</sup> x D<sup>-1</sup> plot) = 0.41 (by regression analysis)m (= q at D = 0 or qS<sub>2</sub>O<sub>3</sub><sup>2-</sup> x D plot) = 0.70 (by regression analysis)Y<sub>g</sub> (average) = 14.43

m (average) = 0.56

TABLE 10

Comparison of Steady State Biomass and Thiosulphate Consumption by  
*T. denitrificans* in

(A) Thiosulphate limited anaerobic culture

(B) Thiosulphate limited aerobic culture

Dilution (h <sup>-1</sup> )	Steady state biomass (mg dry wt. l <sup>-1</sup> )		Steady state thiosulphate consumption (mmol l <sup>-1</sup> )	
	<u>A</u>	<u>B</u>	<u>A</u>	<u>B</u>
0.02	119	211	15.4	19.7
0.03	117	ND	17.0	-
0.04	100	256	17.0	19.7
0.05	123	ND	17.0	-
0.06	142	200	17.0	19.5
0.07	147	ND	17.0	-
0.08	132	250	17.0	19.5
0.10	w	340	-	19.5

Input thiosulphate concentrations were 17mM for A, 20 mM for B.

Mean values from more than one steady state determination.

w = culture wash-out

ND = not determined

three conditions studied (Fig. 22). Over the range 0.02 to 0.08 h<sup>-1</sup> these values ranged from 1.9 to 10.3 mmol h<sup>-1</sup> (g dry wt.)<sup>-1</sup> for the three growth conditions on thiosulphate. Linear regression analysis fits seven or eight values for each growth state indicated m values between 0.56 (aerobic) and 1.2 (anaerobic, nitrate or thiosulphate limited) mmol thiosulphate h<sup>-1</sup> (g dry wt.)<sup>-1</sup> (Table 11).

#### 3.3.8 Responses in anaerobic chemostat cultures to variation of nutrient supplies

Alteration of the input concentration of thiosulphate or nitrate demonstrated that each could be made growth limiting (Table 12). When a large excess of thiosulphate was supplied under nitrate limitation, culture lysis was observed with a low steady state biomass. Supplementation with a small amount of nitrite, allowing more thiosulphate metabolism, partially alleviated this effect.

#### 3.3.9 Elemental composition of *Thiobacillus denitrificans*

Organisms from chemostats operating at five different dilution rates (between 0.02 and 0.08 h<sup>-1</sup>) grown anaerobically and aerobically with thiosulphate limitation or anaerobically with nitrate limitation, all had essentially the same content of carbon, hydrogen and nitrogen as a percentage of the dry weight (Table 13). From fourteen separate analysis the composition [%(w/w) ± S.E.M.] was carbon, 47.40 ± 1.02; hydrogen, 6.88 ± 0.23; nitrogen, 12.70 ± 0.79. The C/N ratios for the three cultures, anaerobic thiosulphate and nitrate limited, and aerobic thiosulphate limited were 3.87, 3.52 and 3.63 respectively. The conclusion that can be drawn from this is that the growth rate and condition did not significantly affect the gross composition of the organism.

#### 3.4 G/C DNA content

The standards employed were *Escherichia coli* and *Micrococcus lysodeikticus* with buoyant densities of 1.7100 and 1.7310 g/cm<sup>3</sup> respectively. The anaerobic nitrate limited *T. denitrificans* sample gave a density between these values of 1.7214 g/cm<sup>3</sup> and a G/C ratio of 63%. Jackson *et al.* (1968), using a similar method, gave a value of

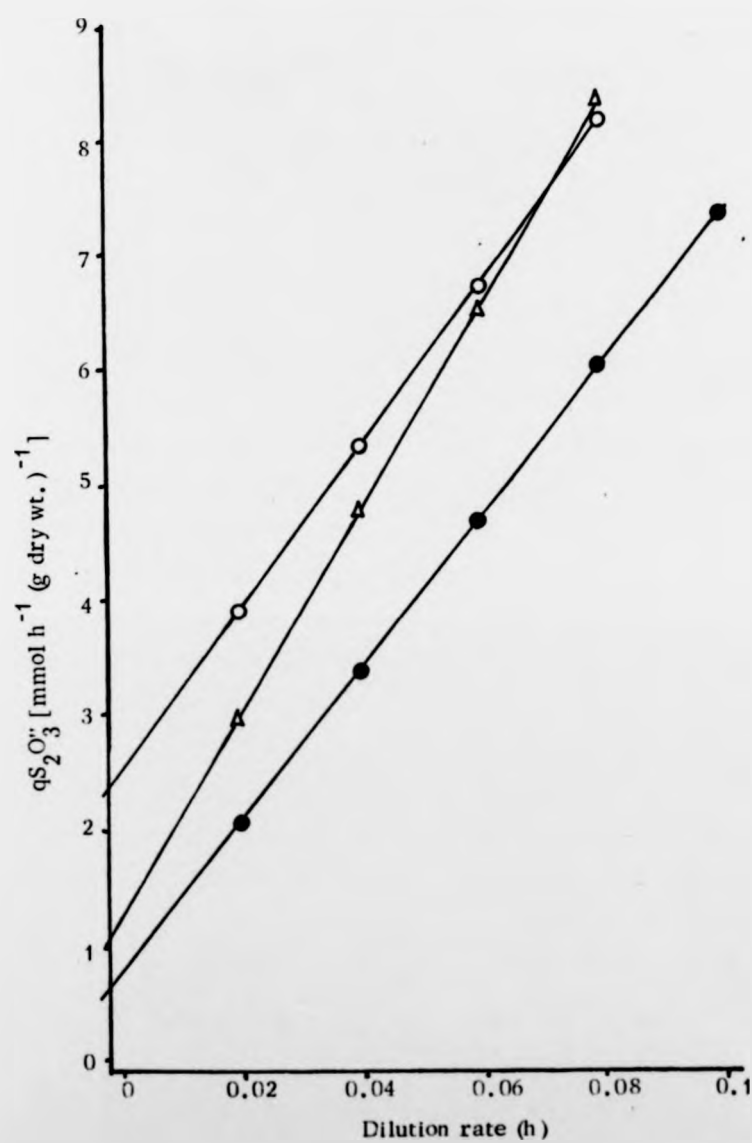


FIG. 22 Comparison of specific rates of thiosulphate consumption of aerobic thiosulphate-limited culture (●), anaerobic thiosulphate-limited culture (○), and anaerobic nitrate limited culture (▲).

TABLE 11

Comparison of  $q\text{S}_2\text{O}_3$  values for  
 (A) nitrate-limited anaerobic,  
 (B) thiosulphate-limited anaerobic  
 (C) thiosulphate-limited aerobic cultures.

Dilution rate ( $\text{h}^{-1}$ )	$q\text{ S}_2\text{O}_3''$ (from results)			$q\text{ S}_2\text{O}_3''$ (by regression analysis)		
	$[\text{mmol h}^{-1} (\text{g dry wt.})^{-1}]$					
	A	B	C	A	B	C
0.02	2.850	2.913	1.99	2.95	3.97	2.43
0.03	3.946	5.379	-	3.85	4.72	-
0.04	4.827	5.913	3.16	4.75	5.42	3.50
0.05	5.519	6.304	-	5.65	6.17	-
0.06	6.609	7.286	5.850	6.52	6.87	4.80
0.07	7.512	7.684	-	7.42	7.67	-
0.08	8.236	7.771	6.92	8.32	8.40	6.15
0.10	-	-	5.735	-	-	7.40

TABLE 12

Effect of input nutrient concentration on steady state conditions and yield of anaerobic cultures of *T. denitrificans* at constant dilution rate ( $0.08 \text{ h}^{-1}$ )

Input concentration (mM)		Steady state concentration (mM)			Biomass ( $\text{mg l}^{-1}$ )	Yield
Thiosulphate	Nitrate	Nitrite	Thiosulphate	Nitrate		
20.14	19.78	0	1.61	0.89	180	9.71
20.14	29.67	0	1.25	2.47	180	9.53
10.07	14.84	0	0.63	0.92	90	9.53
10.07	29.67	0	0	8.78	85	8.44
20.14	29.67	0	1.25	2.97	180	9.53
40.28	29.67	0	21.25	0.59	c.45	c.2.37
40.28	29.67	5.0	16.25	0.69	110	4.58

Between six and eight volume changes of each medium was passed through the culture to establish true steady states before changing to the next medium mixture. Growth yields are expressed as g dry wt. ( $\text{mol thiosulphate}^{-1}$ ).

TABLE 13

Elemental Composition of *T. denitrificans*

<u>Culture condition</u>	<u>%</u>		
	<u>C</u>	<u>H</u>	<u>N</u>
Anaerobic $\text{NO}_3^-$ Ltd, accum.	44.36	6.57	12.41
	44.74	6.75	12.76
Anaerobic $\text{NO}_3^-$ Ltd, $D = 0.06 \text{ h}^{-1}$	47.78	6.68	13.19
	47.85	6.50	13.11
Anaerobic $\text{S}_2\text{O}_3^{2-}$ Ltd, $D = 0.07 \text{ h}^{-1}$	47.46	6.90	13.30
	47.19	6.67	13.37
Anaerobic $\text{S}_2\text{O}_3^{2-}$ Ltd, $D = 0.08 \text{ h}^{-1}$	46.49	6.79	13.60
	46.54	6.90	13.00
Aerobic $\text{S}_2\text{O}_3^{2-}$ Ltd, $D = 0.02 \text{ h}^{-1}$	45.94	6.65	12.80
	45.96	6.74	12.94
Aerobic $\text{S}_2\text{O}_3^{2-}$ Ltd, $D = 0.04 \text{ h}^{-1}$	47.17	7.37	12.63
	47.02	7.27	12.98
Aerobic $\text{S}_2\text{O}_3^{2-}$ Ltd, $D = 0.06 \text{ h}^{-1}$	49.27	6.91	10.96
	49.22	6.99	10.89
Aerobic $\text{S}_2\text{O}_3^{2-}$ Ltd, $D = 0.08 \text{ h}^{-1}$	47.82	6.94	12.52
	47.89	7.06	12.54
Aerobic $\text{S}_2\text{O}_3^{2-}$ Ltd, accum.	43.68	6.05	12.35
	43.07	6.86	12.45

64% and claimed that T. thioparus, T. thiocyanoxidans, T. novellus, T. trautweinii and T. denitrificans were in the same group of G/C ratio of 62-68%. Taylor (personal communication to D.P. Kelly) gave a result of 67.8%.

### 3.5 Check for contamination in chemostat and batch cultures

Low levels of a heterotrophic contaminant occurred in the chemostat studies. This was identified as an Alcaligenes species by the NCIB and was detected on nutrient agar medium III, and thiosulphate agar with supplementary nutrient broth medium II, but was absent from Thiosulphate agar medium I on which T. denitrificans grew abundantly. The concentration of Alcaligenes was at its lowest (0.1%) at high dilution rates in all conditions, but reached as high as 3% in an early anaerobic culture at  $D, 0.02 \text{ h}^{-1}$ . Repeated attempts to separate it completely from T. denitrificans were not successful. It was presumed that the Alcaligenes grew on metabolites secreted by the Thiobacillus, as it was not apparently chemolithotrophic.

### 3.6 Discussion

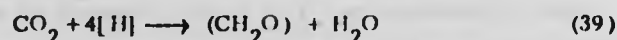
The observations presented demonstrate that T. denitrificans can grow efficiently in chemostat culture under several conditions of nutrient limitation both anaerobically and aerobically. The data obtained for growth yield and maintenance enabled the estimation of the relative growth efficiencies and the amounts of ATP generated by anaerobic and aerobic thiosulphate oxidation.

The anaerobic nitrate-limited culture true growth yield ( $Y_g$ ), of 11.37 g dry wt. (mol thiosulphate)<sup>-1</sup>, can be compared with the observed maximum yield (at  $D = 0.08 \text{ h}^{-1}$ , uncorrected form) of 9.7, and the average yields of 9.3 and 5.7 calculated from the data for batch cultures reported by Taylor et al. (1971) and Lieske (1972). Timmer-ten Hoor, (1976) using a continuous culture at  $D = 0.03 \text{ h}^{-1}$ , obtained 9.28 under similar conditions. In the anaerobic cultures, the growth yields (in terms of g protein or dry wt. per mol thiosulphate consumed) were comparable



with either nitrate or thiosulphate limitation giving  $Y_g$  values of 11.37 and 11.35 respectively (as reported for a single fixed dilution rate of Timmer-ten Hoor, 1976), whereas the yield was higher in aerobic thiosulphate-limited culture, giving  $Y_g$  value of 14.69, as expected from the greater energy available from the aerobic oxidation of thiosulphate (Timmer-ten Hoor, 1976; Kelly, 1978). These  $Y_g$  values are comparable with the higher of two values reported by Hempfling and Vishniac (1967) but are considerably higher than more recently determined values for aerobic T. neapolitanus of 5.27 (Kelly, unpublished) and 6.5 (J.G. Kuenen, personal communication to D.P. Kelly); 7.48 for T. ferrooxidans (Kelly, Eccleston and Jones, 1977; Eccleston and Kelly, 1976, 1978); 5.2 for Thiomicrospira pelophila and about 7 for Thiobacillus A2 (J.G. Kuenen, personal communication to D.P. Kelly). The values for maintenance coefficient ( $m$ ) are comparable with others reported recently (Justin and Kelly, 1976) and from subsequent calculations are equivalent to  $m_{ATP}$  values in the range of 2.62 to 4.4 aerobically and 4.4 to 10.20  $\text{mol h}^{-1} (\text{g dry wt.})^{-1}$  anaerobically. There was indication from some of the data that  $m$  might decrease at lower dilution rates.

Anaerobically, thiosulphate oxidation provides electrons for the energy conserving respiratory chain leading to nitrate reduction. According to equation 31 in the nitrate-limited culture, 7.425 mmol thiosulphate should remain. Electrons, however, are also needed for carbon dioxide reduction to cell carbon, so that the total thiosulphate consumed should be greater than the theoretical nitrate reduction.



On the oxidation of thiosulphate to sulphate, eight reducing equivalents are released which are available for electrons for carbon dioxide fixation, hence  $1\text{CO}_2 = 0.5\text{S}_2\text{O}_3^{2-}$  consumed. The specific amount of carbon dioxide fixed per mol thiosulphate can be calculated

as the carbon content and  $Y_g$  are known. At a carbon content of 47.4% (w/w) of the dry wt., the  $Y_g$  of 11.37 indicates the fixation of 5.39 g carbon or 0.45 mol  $\text{CO}_2$  mol thiosulphate.<sup>-1</sup> As 1  $\text{CO}_2$  requires reducing equivalents from 0.5 mol thiosulphate, then 0.45 mol  $\text{CO}_2 \equiv 0.225 \text{ g mol S}_2\text{O}_3^{2-}$ . So at  $D = \infty$  when  $m = 0$ , then 0.225 g mol thiosulphate consumed is used for carbon dioxide reduction, the rest is for nitrate reduction or other [H] requiring reactions. This can be applied to the specific data obtained. At  $D = 0.06 \text{ h}^{-1}$ ,  $Y = 9.08 \text{ g dry wt. (g mol S}_2\text{O}_3^{2-})^{-1}$ , biomass =  $155 \text{ mg l}^{-1}$ , thio-sulphate consumed =  $17.07 \text{ mmol l}^{-1}$ , nitrate consumed =  $22.75 \text{ mmol l}^{-1}$ .  $155 \text{ mg dry wt.} \equiv 77.5 \text{ mg C} \equiv 6.458 \text{ mol CO}_2$ . As 1  $\text{CO}_2 \equiv 0.5 \text{ mol S}_2\text{O}_3^{2-}$  in [H] demand, then 3.23 mmol thiosulphate consumed to supported 6.458 mol  $\text{CO}_2$  fixation. For the total reduction of nitrate to nitrogen requires 14.22 mmol thiosulphate. So the total theoretical thiosulphate consumption is  $17.45 \text{ mmol l}^{-1}$  which is close to the observed  $17.07 \text{ mmol l}^{-1}$ . The reduction of nitrate to nitrogen takes place via nitrite, nitric oxide and nitrous oxide as intermediates. There was a danger of the gaseous products being lost in the open system so that a complete nitrate reduction would not take place. This evidently did not occur as the theoretical thio-sulphate oxidized was almost reached.

From the same data, the amount of ATP and NADH required for the total process can be assessed. By the Calvin cycle, 1 mol carbon dioxide requires 3 mol ATP and 2 mol NADH to be initially fixed to the level of fructose. Hence 0.45 mol  $\text{CO}_2$  requires 0.9 mol NADH and 1.35 mol ATP. Since the oxidation of 1 mol thiosulphate generates 8 reducing equivalents ([H]), and 0.9 mol NADH requires 1.8 [H] for its formation, only 6.2 [H] are available for energy coupling by the electron transport phosphorylation. Consequently, the observed  $Y_g$  and fixation of 0.45 mol  $\text{CO}_2$  per thiosulphate is supported energetically by the oxidation of 0.775 mol thiosulphate.

Besides  $\text{CO}_2$  fixation by the Calvin cycle, there is an alternative method that accounts for 10%  $\text{CO}_2$  fixation. This is by carboxylation

of phosphoenol pyruvate (derived from phosphoglyceric acid produced by the Calvin cycle). This is a less energy-expensive process as once the phosphoglyceric acid is produced (requires 9 ATP and 6 NADH to produce phosphoglyceric acid from  $3\text{CO}_2$ ), then the fixation of a further molecule by phosphoenol pyruvate requires no more energy. Further ATP is required to effect cell biosynthesis from phosphoglyceric acid, which is no more than 14-20% of the total ATP requirement (Stouthamer, 1973). So the fixation and conversion of 0.45 mol  $\text{CO}_2$  to the level of cell constituents also requires 0.39 mol ATP for biosynthesis from the hexose level, and the reduction of 0.9 mol NAD probably requires 1.8 mol ATP to effect electron transport from the level of cytochrome c to  $\text{NAD}^+$ . This is assuming that 2 mol ATP is required to reduce 1 mol  $\text{NAD}^+$ . Consequently, the total ATP indicated to be available from 0.775 mol thiosulphate was 3.54 mol, i.e. 4.57 mol ATP per thiosulphate oxidized for energetic purposes with nitrate as the terminal acceptor. The energy requirement for  $\text{NAD}^+$  reduction in thiosulphate oxidizing thiosulphate has been found to be up to 2 or 3 ATP per  $\text{NAD}^+$ . This is consistent with an "energy gap" of 100 kJ between cytochrome c and  $\text{NAD}^+$  (Kelly, 1978), although lower values of 1 or 1.3 ATP/ $\text{NAD}^+$  have been claimed (Hempfling and Vishniac, 1967; Ross et al., 1968; Aleem, 1970; Cole and Aleem, 1970; Tuovinen and Kelly, 1972). If the requirement for the production of each NADH is 1, 2 or 3 mol ATP in T. denitrificans, then the total ATP available from oxidizing 0.775 mol thiosulphate would be 2.64, 3.54 or 4.44 mol, i.e. 3.41, 4.57 or 5.73 mol ATP per thiosulphate oxidized. The results from anaerobic thiosulphate-limited culture are very similar to these as the  $Y_g$  was 11.37 g dry wt. g mol thiosulphate<sup>-1</sup>.

Aerobically, the ATP content can be calculated in the same manner. At a carbon content of 47.4% (w/w) of the dry wt. and the  $Y_g$  of 14.43, 6.84 carbon or 0.57 mol carbon dioxide are fixed per mol thiosulphate oxidized, of which 0.285 g mol thiosulphate consumed is

used for carbon dioxide reduction. 0.57 mol  $\text{CO}_2$  requires 1.71 mol ATP and 1.14 mol NADH to be fixed to the level of fructose. The NADH requires 2.28 [H] for its formation, so only 5.72 [H] are available for energy coupling by the electron transport phosphorylation. Therefore, the Yg and fixation of 0.57 mol  $\text{CO}_2$  per thiosulphate is supported energetically by oxidation of 0.715 mol thiosulphate. The fixation and conversion of 0.57 mol carbon dioxide to the level of cell constituents requires 0.49 mol ATP for biosynthesis from the hexose level. Consequently, the total ATP indicated to be available (on the assumption that 2 ATP is required per  $\text{NAD}^+$  reduced) from 0.715 mol thiosulphate was 4.48 mol, i.e., 6.27 mol ATP per mol thiosulphate oxidized for energetic purposes with oxygen as the terminal electron acceptor. If the requirement for NADH production is 1, 2 or 3 ATP, then the total ATP required per thiosulphate oxidized would be 4.67, 6.27 or 7.86 compared with 3.41, 4.57 and 5.73 in anaerobic conditions. If the values assuming 2 ATP/NADH produced are taken and it is assumed that they represent 6 to 7 mol ATP formed per thiosulphate aerobically and 4 to 5 mol ATP anaerobically, it is possible to deduce that 1 to 2 mol ATP are formed in each case by substrate phosphorylation (Peck, 1968), and at least 5 and 3 mol ATP are formed by oxidative phosphorylation aerobically and anaerobically respectively.

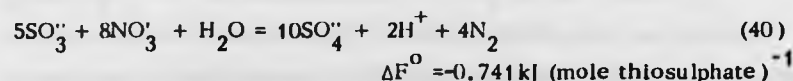
Timmer-ten Hoor (1976) obtained similar yield results, but her overall calculation led to 6.63 ATP per mole thiosulphate anaerobically and 10.59 ATP aerobically. Her method of calculation was based on a gross formula of the cell material of  $\text{C}_{40}\text{H}_{74}\text{O}_{17}\text{N}_9$  and hence an estimation of gram equivalent of electrons needed for its production, leading to  $Y_{\text{ATP}}$  values in a similar manner to Stouthammer and Bettenhausen (1973). From this she estimated the reducing equivalents available for energy coupling by the electron transport phosphorylation to be 6.38 anaerobically and 5.71 aerobically, as compared to 6.2 and 5.72 found here, respectively. Her ATP

calculations were based on starting with  $Y_g$  values corrected for energetic purposes, whereas the calculations included here were started from the experimental  $Y_g$  results and finally corrected to include the electrons required to fix carbon dioxide.

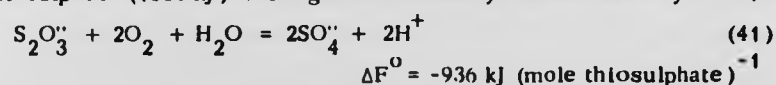
By using  $Y_g$  values around 7 for *T. ferrooxidans* growing on thio-sulphate (Kelly *et al.*, 1977, Eccleston and Kelly, 1978) the aerobic thiosulphate oxidation supported synthesis of only 3 mol ATP per mol, and that while 2 mol ATP could be produced by electron transport phosphorylation, it was possible that the oxidation of the sulphane-sulphur of thiosulphate was not energy conserving. This would be consistent with the operation of a non-energy-linked oxygenase system in aerobic thlobacilli for the conversion of sulphur to sulphide (Suzuki and Silver, 1966; Silver and Lundgren, 1968; Taylor, 1968). The presence or absence of the oxygenase reaction in sulphur oxidation is of great significance in assessing the bioenergetic capabilities of thlobacilli as some of the oxygen consumed will be incorporated in converting sulphur to sulphite and not used in the oxidation of the electron transport chain, thus reducing the available energy yield of the oxidation. The considerably higher  $Y_g$  values reliably reported on *T. denitrificans* here and previously (Justin and Kelly, 1976; Timmer-ten Hoor, 1976) and the calculated ATP values, indicates that the oxidation of the sulphane-sulphur of thiosulphate supports phosphorylation and hence is not affected by the sulphur oxygenase which would not be expected to be significant in anaerobic growth.

The calculations indicate that for each mol thiosulphate used for energetic purposes, i.e. corrected for [H] requirement for carbon dioxide fixation, the amount of growth supported is 14.67 g dry wt. under anaerobic nitrate and thiosulphate limitation. Anaerobically, the available energy would seem to be 72.70% of that available aerobically. This is compared to Timmer-ten Hoor (1976) with 11.60 g dry wt. and 18.54 g dry wt. g mol thiosulphate<sup>-1</sup> respectively, giving a figure of 62.57%.

An alternative means of calculating the ATP production would be by the thermodynamic calculation of the reactions free energy production (Kelly, 1978). This would only be possible if the reaction was 100% efficient. With a complex reaction of thiosulphate and nitrate or oxygen, the reactions are not reliable as the oxidations can be broken down into a number of steps, each giving some energy release, but some of these may be coupled to energy conservation through ATP synthesis. The estimation of the total overall efficiency of conversion of energy from oxidation reactions to metabolic energy as measured by the production of new bacteria growth should be possible. Carbon dioxide converted to  $C_6H_{12}O_6$  requires  $495 \text{ kJ mole}^{-1}$ . The efficiency of 1 mol of  $CO_2$  by T. denitrificans can be calculated from the Yg figures.



In the nitrate limited anaerobic culture, the Yg of 11.35 g dry wt. g mol thiosulphate<sup>-1</sup> and 0.45 mol  $CO_2$  fixed per thiosulphate = 2.22 mol thiosulphate (1646.67 kJ) to fix one mol  $CO_2$  (495 kJ) giving an overall efficiency of 30.06%. This includes the requirement for reducing equivalents, and is close to the 32% obtained by Timmer-ten Hoor (1976). Aerobically T. denitrificans fixes one mol  $CO_2$  at the expense of 1.75 mol thiosulphate (1638 kJ) which gives a thermodynamic efficiency of 30.22%.



Earlier reports on T. denitrificans give efficiencies of 8.7% (anaerobic) by Lieske (1912) and 9% by Baas-Becking and Parks (1927) on batch cultures grown for several weeks. Baalsrud and Baalsrud (1952) used short term cultures and found a maximum efficiency of 25%. By comparison, T. ferrooxidans fixes one  $CO_2$  per 18.8  $Fe^{2+}$  (620 kJ) with 80% efficiency. When this is corrected for [H] requirement,  $CO_2$  fixation is supported by 14.4  $Fe^{2+}$  with a thermodynamic efficiency of 100%.

probably indicating underestimation of  $-\Delta F$  for iron oxidation at pH 1.5 (Kelly, 1978). The low efficiency in *T. denitrificans* may indicate that some intermediate oxidation steps, while being exergonic, are not coupled to energy conserving processes, consequently lowering the maximum efficiency possible.

In comparison, for the theoretically available free energy for nitrate-linked and oxygen-linked thiosulphate oxidation (741 and 963 kJ mol<sup>-1</sup> respectively), the energy available anaerobically seems to be no greater than 79.2% of that available aerobically, which is in moderate agreement with the observed results of 72.7%.

The anaerobic nitrate limited values for thiosulphate (11.37 g dry wt. (g mol S<sub>2</sub>O<sub>3</sub><sup>2-</sup>)<sup>-1</sup>), and tetrathionate (21.34 g dry wt. (g mol S<sub>4</sub>O<sub>6</sub><sup>2-</sup>)<sup>-1</sup>) are equivalent to, respectively, 14.66 and 28.24 g dry wt. per mol thiosulphate or tetrathionate oxidized for energetic purposes, indicating that the energy available from thiosulphate oxidation is 51.9% of that available from tetrathionate oxidation. This is in reasonable agreement with the figure for the relative free energy available from their oxidation of 54-56% (Kelly, 1978).



PART 4: METABOLIC CHANGES ACCOMPANYING THE  
TRANSITION OF T. DENITRIFICANS FROM  
AEROBIC TO ANAEROBIC GROWTH IN  
CONTINUOUS CHEMOSTAT CULTURE



PART 4: METABOLIC CHANGES ACCOMPANYING THE TRANSITION OF  
T. DENITRIFICANS FROM AEROBIC TO ANAEROBIC GROWTH  
IN CONTINUOUS CHEMOSTAT CULTURE

4.1 Introduction

Thiobacillus denitrificans is the only well established facultatively anaerobic Thiobacillus and is capable of rapid growth both aerobically and anaerobically with nitrate, nitrite or nitrous oxide, as oxidant. No study has been reported on the biochemical and physiological changes accompanying the transition of the organism from aerobic to anaerobic conditions, including the enzymes involved in the nitrate reduction. The claim that prolonged aerobic cultures resulted in loss of capacity to grow anaerobically has never been reliably substantiated (Vishniac and Santer, 1957; Woolley, Jones and Happold, 1962).

In the previous chapter it was speculated that during aerobic growth of T. denitrificans more energy was conserved during thiosulphate oxidation than during growth of other aerobic thiobacilli (Eccleston and Kelly, 1978; Justin and Kelly, 1978). If energy was conserved during the oxidation of the sulphane-sulphur of thiosulphate by T. denitrificans, but not when this is oxidized by other thiobacilli such as T. ferrooxidans (Eccleston and Kelly, 1978), then a larger aerobic growth yield could be possible. This could occur if the sulphur-oxidizing oxygenase enzyme found in aerobic thiobacilli (Suzuki, 1965a, b; Suzuki and Silver, 1966; Charles and Suzuki, 1966; Silver and Lundgren, 1968a), postulated to play a central role in sulphane-sulphur oxidation (Kelly, 1968; Roy and Trudinger, 1969), was not important in aerobic oxidation of T. denitrificans. This enzyme would not be expected to be present during anaerobic growth.

The initial cleavage of thiosulphate to sulphite and sulphide can be carried out in two ways by either a one-step reaction splitting the thiosulphate with a thiol requiring enzyme (Aminuddin, 1974; Aleem, 1977), or be converted to tetrathionate and subsequent reductive

cleavages to thiosulphate, sulphur and sulphite. For the latter to take place, the thiosulphate-oxidizing enzyme must be present. The presence of this enzyme was investigated in this section.

#### 4.2 Effect of dissolved oxygen concentrations on biomass, substrate consumption and yield in steady state chemostats

Using chemostat cultures at a fixed dilution rate of  $0.08 \text{ h}^{-1}$  with a medium containing about 19 mM thiosulphate and about 24 mM nitrate, a series of steady states was established at different dissolved oxygen concentrations over a period of 2334.75 h. The aerobic culture was established at  $0.08 \text{ h}^{-1}$  after 1005 h before the drop in dissolved oxygen concentration was carried out. The monitored percentages of dissolved oxygen of air saturation were calculated as  $\mu\text{M-O}_2$ . This was based on 100% air saturation of water being equal to  $245 \mu\text{M-O}_2$  (Tuovinen and Kelly, 1972).

The consequence of progressively decreasing the steady state dissolved oxygen from  $216 \mu\text{M-O}_2$  in a fully aerobic culture to  $12 \mu\text{M-O}_2$  was to increase the steady state biomass without decreasing consumption of the limiting nutrient, thiosulphate, which was completely consumed at all dissolved oxygen concentrations. Fig. 23 shows the change of biomass with changes of dissolved oxygen in the culture vessel. Consequently the yield increased with decreasing steady state dissolved oxygen, while the specific rate of the thiosulphate oxidation ( $q\text{S}_2\text{O}_3^{2-}$ ) decreased (Table 14).

At all dissolved oxygen concentrations tested, there was no significant reduction of nitrate, although trace amounts of nitrite were formed at 22 to  $12 \mu\text{M-O}_2$  (Table 14). The change from  $12 \mu\text{M-O}_2$  thiosulphate limited aerobic to nitrate limited anaerobiosis resulted in immediate nitrate reduction and transitory accumulation of sulphur and nitrite within 4 h. When the steady state was established, sulphur was absent and nitrite negligible. About 2.6 mM thiosulphate remained unconsumed, which was as expected. Re-aerating the anaerobic nitrate limited culture to establish  $192 \mu\text{M-O}_2$ , i.e. aerobic thiosulphate limited, resulted in complete consumption of thiosulphate and suppression of nitrate reduction.

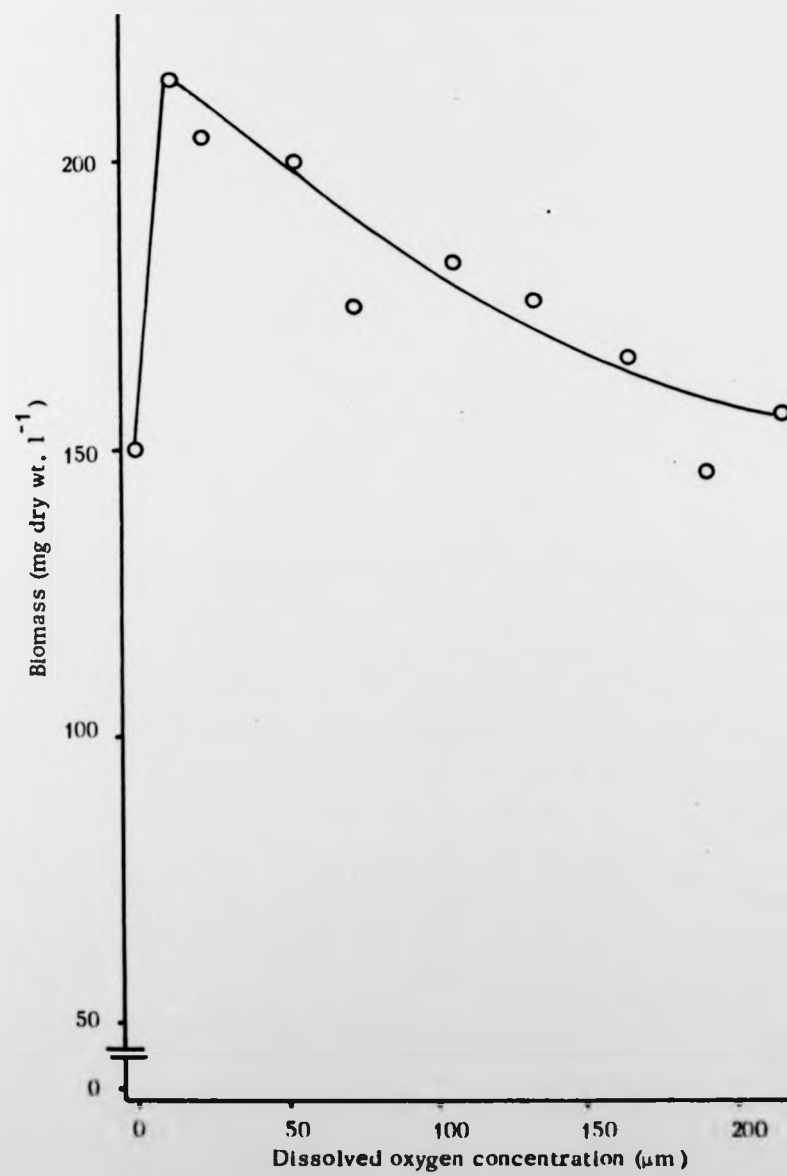


FIG. 23 Biomass vs. dissolved oxygen concentration.

TABLE 14 Effect of dissolved oxygen concentration on biomass, substrate consumption and yield in steady state chemostat cultures of *Thiobacillus denitrificans* at  $D$ ,  $0.08 \text{ h}^{-1}$

Dissolved oxygen concentration <sup>a</sup> ( $\mu\text{M}$ )	Biomass (mg dry wt $\text{l}^{-1}$ )	Thiosulphate consumed ( $\text{mmoles l}^{-1}$ )	Yield (g dry wt (g mol $\text{Na}_2\text{S}_2\text{O}_3$ ) <sup>-1</sup> )	$q_{\text{S}_2\text{O}_3}$ ( $\text{mmol (g dry wt)}^{-1} \text{ h}^{-1}$ )	Nitrate consumed ( $\text{mmol l}^{-1}$ )	Nitrite produced ( $\text{mmol l}^{-1}$ )
216	155	18.69	7.75	10.3	0	0.015
191	145	18.69	7.38	10.8	0	0.020
167	165	18.69	7.57	10.6	0	0.013
135	175	18.69	10.06	8.0	0	0.012
108	182	18.69	9.82	8.1	0	0.011
74	175	18.69	10.54	7.6	1.1	0.021
54	200	18.69	10.58	7.6	0	0.030
22	205	18.69	11.36	7.0	0	0.117
12	215	18.69	12.34	6.5	0	0.156
0	150	16.06	9.40	8.5	23.4	0.171

<sup>a</sup> Air-saturated medium contained  $245 \mu\text{M O}_2$ . Dissolved  $\text{O}_2$  concentration was calculated from monitoring dissolved oxygen in terms of % air saturation levels.

At a later date an immediate change from aerobic thiosulphate-limited at  $0.08 \text{ h}^{-1}$  to anaerobic thiosulphate-limited culture was carried out. No sulphur deposited nor was any nitrite accumulated, but a steady washout proceeded. This was stopped when the anaerobic culture was changed from  $0.08 \text{ h}^{-1}$  to  $0.07 \text{ h}^{-1}$  where all the thiosulphate was consumed and 1.68 mM nitrate remained.

#### 4.3 Nitrite reduction by suspensions of organism grown with different concentrations of dissolved oxygen

Organisms were harvested from steady states held at eleven different dissolved oxygen concentrations. These were tested for their ability to reduce nitrite anaerobically in the presence of thiosulphate. The ability of the organisms to react in anaerobic conditions increased with the drop of dissolved oxygen in the culture from which the organisms were harvested. Organisms from cultures held at  $167 \mu\text{M-O}_2$  and above did not reduce nitrite anaerobically during 5 h incubation at  $30^\circ \text{C}$ . Organisms grown at lower dissolved oxygen concentrations reduced nitrite after a lag, the length of which was a function of the dissolved oxygen regime in the culture from which the organisms were harvested (Fig. 24). There was no lag with organisms grown anaerobically with nitrate limitation. The thiosulphate oxidation also proceeded after a lag period which increased in length with the increase of dissolved oxygen, except under complete anaerobiosis where thiosulphate was immediately oxidized (Fig. 25). No tetrathionate accumulated during this period.

#### 4.4 Nitrate reduction by suspension of organism grown with different concentrations of dissolved oxygen

Organisms harvested for the previous section were used at the same time to follow the rate of nitrate reduction when subjecting them to complete anaerobic conditions. Only organisms grown fully anaerobically in nitrate-limited cultures showed complete nitrate reduction activity (Fig. 26). All other organism grown at dissolved

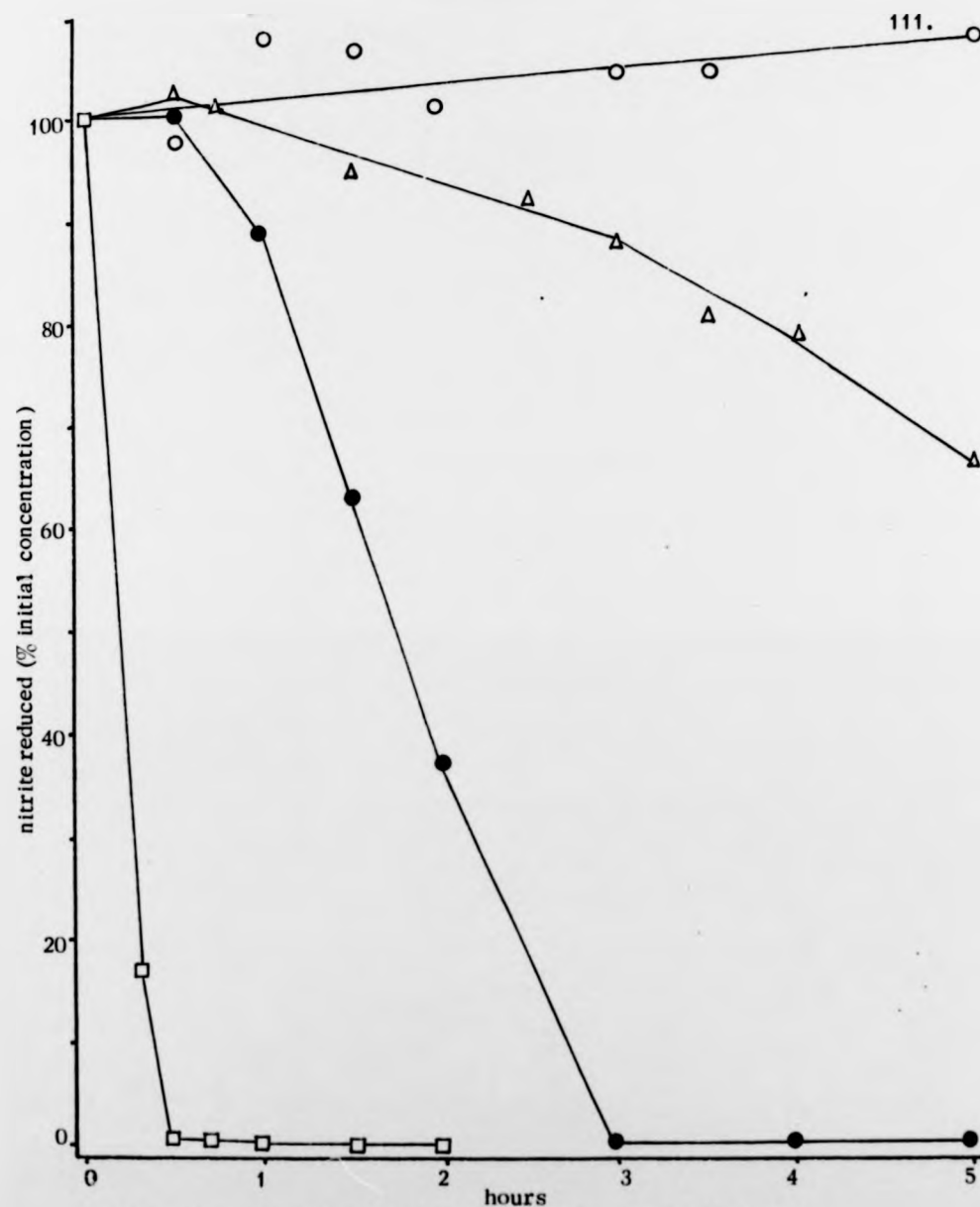


FIG. 24 Nitrite reduction by anaerobic suspensions of *Thiobacillus denitrificans* taken from chemostat cultures grown with different concentrations of dissolved oxygen. Consumption of nitrite is expressed as % of initial 1.8 mM  $\text{NaNO}_2$  in the presence of 2 mM  $\text{Na}_2\text{S}_2\text{O}_3$ . Steady state oxygen concentrations in the cultures from which the bacteria were taken were 216  $\mu\text{M}$ ; (O) 135  $\mu\text{M}$ ; ( $\Delta$ ) 74  $\mu\text{M}$ ;  $\bullet$  0. ( $\square$ )

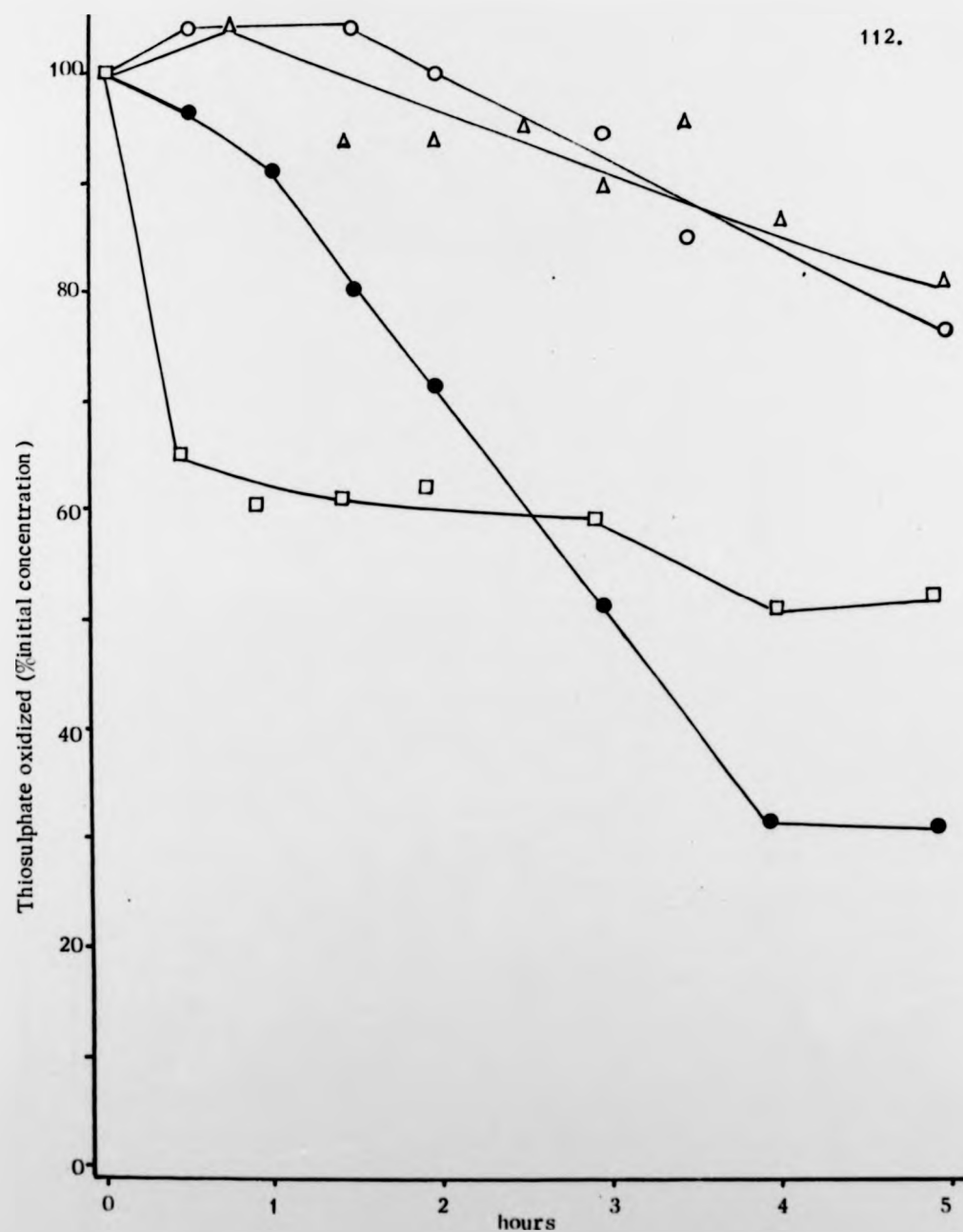


FIG. 25 Thiosulphate oxidation by anaerobic suspensions of *T. denitrificans* grown on nitrite and thiosulphate, taken from chemostat cultures grown with different concentrations of dissolved oxygen, 216 μM (○); 135 μM (Δ); 74 μM (●); 0 (□). Consumption of thiosulphate expressed as % of initial 2 mM Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>.

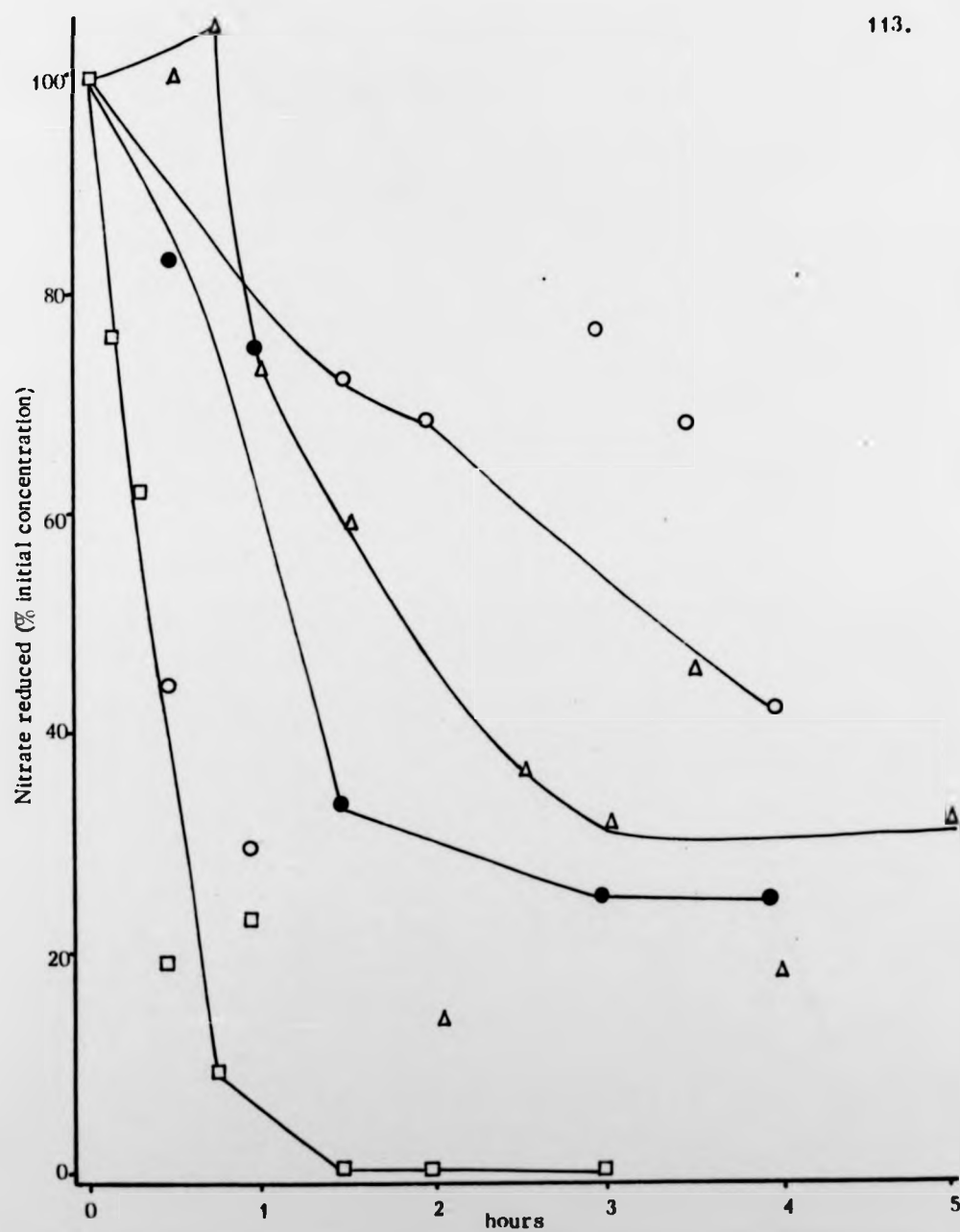


FIG. 26 Nitrate reduction by anaerobic suspensions of *T. denitrificans* grown on nitrate and thiosulphate taken from chemostat cultures grown with different concentrations of dissolved oxygen: 216  $\mu\text{M}$  (O), 135  $\mu\text{M}$  ( $\Delta$ ), 74  $\mu\text{M}$  ( $\bullet$ ), 0  $\mu\text{M}$  ( $\square$ ). Consumption of nitrate expressed as % of initial 2 mM  $\text{KNO}_3$ .



oxygen between 132 and 12.48  $\mu\text{M-O}_2$  showed a small reduction of nitrate which did not lead to completeness. At the same time the rate of thiosulphate oxidation increased, the lower the dissolved oxygen in the culture vessel, with a decrease in lag until at 12.48  $\mu\text{M-O}_2$  and complete anaerobiosis there was no lag at all (Fig. 27). No tetrathionate was produced during this reaction.

Organisms at 163.2  $\mu\text{M-O}_2$  or above did not produce any nitrite. From 163.2  $\mu\text{M-O}_2$  to complete anaerobiosis, varying amounts of nitrite were produced. The lower the dissolved oxygen that the organism was grown in, the greater the amount of nitrite produced. The nitrite produced reached a peak early on in the incubation period with its uptake being a function of the dissolved oxygen (Fig. 28). The use of this nitrite was almost instant.

#### 4.5 Nitrate and nitrite reductase activities

Nitrate and nitrite reductases were assayed in crude extracts using NADH as reductant. The activities of both enzymes were negligible in cultures maintained <sup>with</sup> dissolved oxygen concentrations equivalent to 127  $\mu\text{M-O}_2$  and above (Table 15). Nitrate reductase increased slightly below 54  $\mu\text{M-O}_2$  and to 12 times the aerobic level under complete anaerobiosis, from 1.45 (mean value of six results) to 18.9  $\text{nmol min}^{-1} (\text{mg protein})^{-1}$ . Nitrite reductase activity increased considerably as the dissolved oxygen concentration was lowered from 91 to 14  $\mu\text{M-O}_2$  but rose to very high levels of 269  $\text{nmol min}^{-1} (\text{mg protein})^{-1}$  in anaerobic cultures (Table 15). Using freshly harvested anaerobic organisms, grown on nitrate-limited media, assayed soon after breaking in the French pressure cell, the maximum nitrate reductase activity was 28.6  $\text{nmol min}^{-1} (\text{mg protein})^{-1}$  and nitrite reductase activity was 264.0  $\text{nmol min}^{-1} (\text{mg protein})^{-1}$ .

The conversion of aerobic thiosulphate-limited culture to anaerobic thiosulphate-limited resulted in the increase of nitrate reductase from 1  $\text{nmol min}^{-1} (\text{mg protein})^{-1}$  to 10  $\text{nmol min}^{-1} (\text{mg protein})^{-1}$ , and nitrite reductase activity from none under aerobic

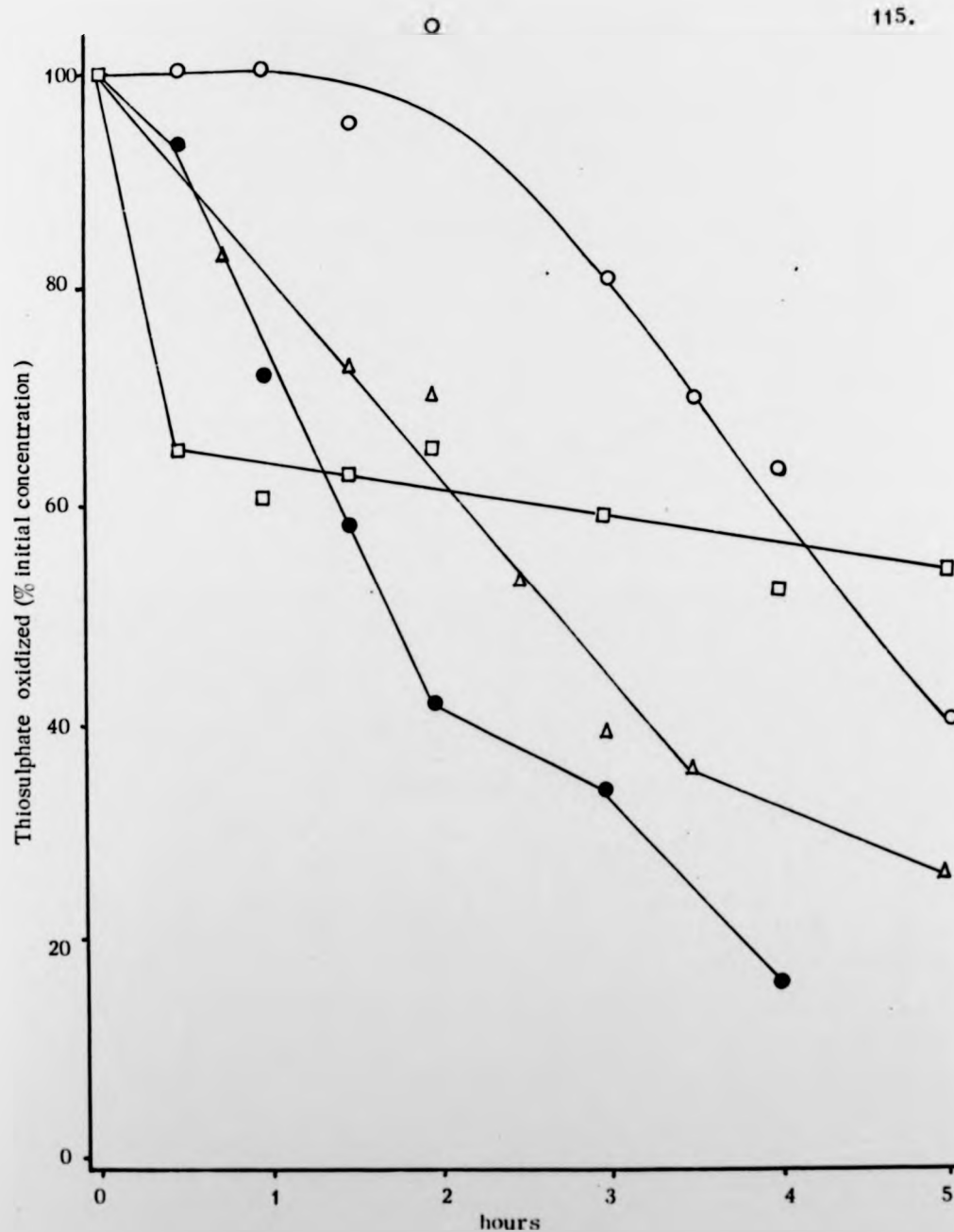


FIG. 27 Thiosulphate oxidation by anaerobic suspensions of *T. denitrificans* grown on nitrate and thiosulphate, taken from chemostat cultures grown with different concentrations of dissolved oxygen: 216 μM (○); 135 μM (Δ); 74 μM (●), 0 μM (□). Consumption of thiosulphate expressed as % of initial 2 mM Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>.

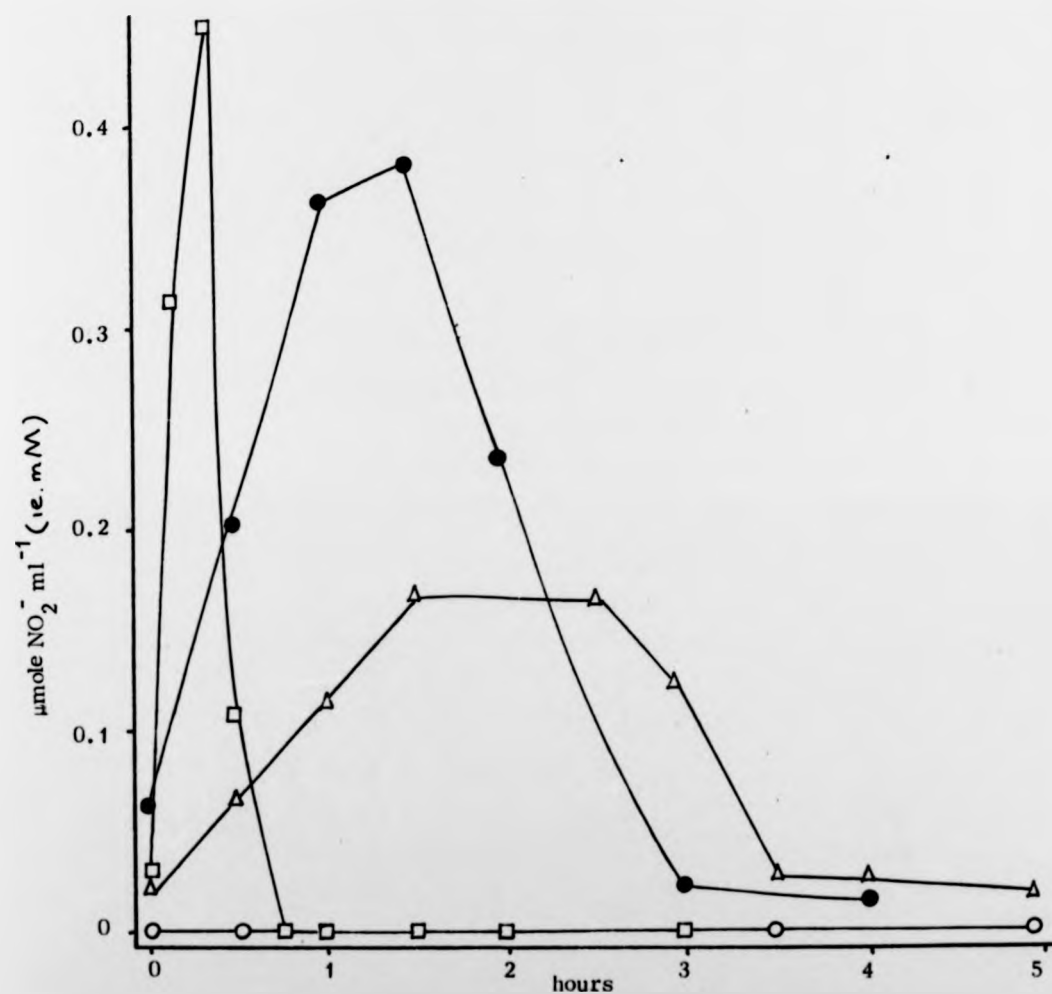


FIG. 28 Nitrite production by anaerobic suspensions of *T. denitrificans* grown on nitrate and thiosulphate taken from chemostat cultures grown with different concentrations of dissolved oxygen: 216 µM (O), 135 µM (Δ), 74 µM (●), 0 µM (□).

TABLE 15 Nitrate and nitrite reductase activities in crude  
extracts of *Thiobacillus denitrificans* from chemostat  
cultures at  $D, 0.08 \text{ h}^{-1}$

Dissolved oxygen concentration ( $\mu\text{M}$ )	Growth- limiting substrate	Enzyme activity	
		(nmol min <sup>-1</sup> (mg protein) <sup>-1</sup> )	
		Nitrate reductase	Nitrite reductase
211	Thiosulphate	1.6	1.2
176	Thiosulphate	1.3	1.3
147	Thiosulphate	1.3	0
127	Thiosulphate	1.7	0
91	Thiosulphate	1.2	29.4
86	Thiosulphate	1.6	61.7
54	Thiosulphate	2.2	48.5
15	Thiosulphate	4.0	85.9
14	Thiosulphate	9.0	47.4
0	Nitrate	18.9	269.0
0	Nitrite	2.0	280.0
0	(nitrate free medium)		
0	Nitrate	15.8	241.0
0	(K <sub>2</sub> S <sub>4</sub> O <sub>6</sub> instead of Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> )		
0	Thiosulphate	10.0	82.0
0	Thiosulphate	7.8	77.1
	(N <sub>2</sub> O instead of NO <sub>3</sub> <sup>-</sup> )		

conditions to  $82 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$  under anaerobiosis. These anaerobic results are lower than those obtained from nitrate-limited cultures.

The nitrate reductase was not raised above its aerobic levels in anaerobic organisms cultured in nitrate-free nitrite limited medium, but nitrite reductase reached a level comparable to that under nitrate limited anaerobiosis (Table 15). Substituting tetrathionate for the usual thiosulphate substrate under anaerobic nitrate-limitation resulted in enzyme activities comparable to those in thiosulphate cultures (Table 15).

Both nitrate and nitrite reductases were found present in the organism grown with nitrous oxide, replacing nitrate, in thiosulphate-limited culture. The activities of these enzymes were similar to those in the anaerobic thiosulphate-limited culture at  $7.83 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$  for nitrate reductase and  $77.05 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$  for nitrite reductase. Both thiosulphate cultures grown on nitrous oxide and nitrate were freshly harvested and assayed soon after breaking the cells.

The activity of the enzymes from cells stored at  $4^{\circ} \text{C}$  was recovered by the addition of phenazine methosulphate. This is in agreement with the findings of Sawhney and Nicholas (1977).

#### 4.6 Sulphur-oxidizing enzyme

The activities of the sulphur-oxidizing enzyme were found to be very low in both aerobic and anaerobically grown organisms (Table 16). In most cases the molar ratio of oxygen consumed to thiosulphate formed was about 1.0, as required by the mechanism proposed by Suzuki (1965a, b) and Suzuki and Silver (1966). Freshly prepared crude extracts from freshly harvested aerobic ( $196 \mu\text{M-O}_2$ ) and anaerobic cultures gave activities of  $1.069$  and  $0.228 \mu\text{mol thiosulphate formed (210 min)}^{-1} (\text{mg protein})^{-1}$ , respectively. The activity in crude extracts declined as the dissolved oxygen content of the cultures was lowered (Table 16). Mean values for six aerobic cultures (127 to

TABLE 16 Sulphur-oxidizing enzyme activity in chemostat cultures of *Thiobacillus denitrificans* grown with different dissolved oxygen concentrations

Dissolved oxygen concentration ( $\mu\text{M}$ )	Growth-limiting substrate	Enzyme activity	
		( $\mu\text{mol (210 min)}^{-1}$ Oxygen uptake)	( $\text{mg protein}^{-1}$ ) Thiosulphate formation
211	Thiosulphate	0.42	0.44
176	Thiosulphate	0.61	0.63
147	Thiosulphate	0.50	0.31
127	Thiosulphate	0.39	0.92
91	Thiosulphate	0.47	0.45
86	Thiosulphate	0.51	0.24
54	Thiosulphate	0.28	0.11
22	Thiosulphate	0.35	0.40
15	Thiosulphate	0.31	0.36
14	Thiosulphate	0.14	0.14
0	Nitrate	0.18	0.17
0	Nitrite	0.41	0.16
	(Nitrate-free medium)		
0	Nitrate	0.76	0.25
	( $\text{K}_2\text{S}_4\text{O}_6$ instead of $\text{Na}_2\text{S}_2\text{O}_3$ )		

211  $\mu\text{M-O}_2$ ) and four anaerobic cultures were  $0.740 \pm \text{S.E.M. } 0.32$  and  $0.202 \pm 0.038 \mu\text{mol thiosulphate formed (210 min)}^{-1} (\text{mg protein})^{-1}$ .

Activity was dependent on the presence of both cell-free extract and reduced glutathione. In the absence of cell free extracts but with the complete reaction mixture, no thiosulphate was produced but  $0.32 \mu\text{mol oxygen}$  was consumed, whereas in the absence of cell free extracts and reduced glutathione  $0.20 \mu\text{mol oxygen}$  was consumed and  $0.27 \mu\text{mol thiosulphate produced (210 min)}^{-1}$ . In one test with an aerobic extract,  $1.20 \mu\text{mol thiosulphate (210 min)}^{-1} (\text{mg protein})^{-1}$  was formed by the complete assay mixture and  $0.27 \mu\text{mol}$  in the absence of glutathione. This could indicate some glutathione independent activity, perhaps comparable with that studied by Taylor (1968) on *T. neapolitanus*, except that  $0.27 \mu\text{mol thiosulphate}$  was also produced in a reaction mixture lacking in cell free extracts. So the mechanism suggested by Suzuki (1965a, b) and Suzuki and Silver (1966) possibly occurs at low levels in *T. denitrificans*.

#### 4.7 Thiosulphate-oxidizing enzyme

Thiosulphate-oxidizing enzyme was detected in all steady state cultures from 211  $\mu\text{M-O}_2$  to anaerobiosis. The activities in two freshly harvested and assayed aerobic cultures (196  $\mu\text{M-O}_2$ ) were  $0.144$  and  $0.112 \mu\text{mol ferricyanide reduced min}^{-1} (\text{mg protein})^{-1}$ , whereas in comparable anaerobic culture it was only  $0.018$ . The aerobic cultures were thiosulphate limited, and the anaerobic culture was nitrate limited. Activity was typically  $0.012$  to  $0.02 \mu\text{mol ferricyanide reduced min}^{-1} (\text{mg protein})^{-1}$  in all anaerobic cultures. Activity was gradually lost on storage of the cells and cell free extracts at  $-20^\circ\text{C}$ . Activity in freshly harvested and broken cells was  $0.144 \mu\text{mol ferricyanide reduced min}^{-1} (\text{mg protein})^{-1}$  but only  $0.014$ - $0.029$  in extracts made from organisms stored 158-205 days before use and then stored 2-7 days at  $-20^\circ\text{C}$ .

These low activities could be compared with the crude extract activities [ $\mu\text{mol ferricyanide reduced min}^{-1} (\text{mg protein})^{-1}$ ] of  $4.97$  for *T. neapolitanus* (Kelly, 1966),  $9.00$  for *T. ferrooxidans* (Silver

and Lundgren, 1968a, b) and 0.96 for T. thioparus (Lyric and Suzuki, 1970). T. neapolitanus and T. ferrooxidans have a pH optimum of 5.0 with little or no activity at pH values above 7.5, whereas T. thioparus was active over a wide range of pH (4.5-10.00), though by using ferricyanide acceptor the optimum pH was 5.0. These assays were carried out on T. denitrificans at pH 5.0 after a comparison of methods using potassium phthalate buffer, pH 5.0 (Trudinger, 1961) and a phosphate buffer, pH 7.4 (Lyric and Suzuki, 1970). Activity at pH 5.0 was greater than at 7.4 (Table 17), as demonstrated by Trudinger (1961) on Thiobacillus X where the rate goes up as pH is lowered from 7.1 to 4.5, at which point a chemical reaction occurs.

#### 4.8 Discussion

The mechanism of sulphur-compound-dependent nitrate reduction in T. denitrificans is basically similar to that in other anaerobic nitrate reducing bacteria: Pseudomonas denitrificans (Adams, Warnes and Nicholas, 1971), Pseudomonas perfectomarinus and Alcaligenes faecalis (Ishaque and Aleem, 1973; Eldensperger and Garcia, 1975), Micrococcus denitrificans (Sawhney and Nicholas, 1977). However, unlike Pseudomonas fluorescens and Pseudomonas chlorophis which produce nitrous oxide as their gaseous end product of denitrification (Greenberg and Becker, 1977). T. denitrificans is a complete reducer taking nitrate all the way to nitrogen.

The regulation of this system under aerobic and anaerobic chemostat conditions with thiosulphate, tetrathionate, nitrate, nitrite and nitrous oxide has not previously been reported. This is the first comprehensive study of nitrate, nitrite and nitrous oxide reduction in a chemostat system by T. denitrificans. The presence of nitrate reductase and nitrite reductase in T. denitrificans has been thoroughly investigated in anaerobic conditions with thiosulphate and nitrate only (Sawhney and Nicholas, 1977, 1978a, b), or sulphide and nitrite aerobically and anaerobically (Aminuddin and Nicholas (1973).



TABLE 17 Comparison of thiosulphate-oxidizing enzyme activity at pH 5.0 and pH 7.4

Condition			Specific activity ( $\mu\text{mol Fe(CN)}_6^{3-}$ reduced $\text{min}^{-1}$ ( $\text{mg protein}^{-1}$ ))	
Dissolved oxygen concentration ( $\mu\text{M}$ )	Growth - limiting substrate	Respiratory oxidant	pH 5.0	pH 7.4
0	Thiosulphate	Nitrate	0.0045	0.0015
0	Thiosulphate	Nitrous oxide	0.0017	0.0011

The experiments in this chapter demonstrate that the levels of NADH-dependent nitrate and nitrite reductase are regulated in response to oxygen tension, significant activities of nitrate reductase being detected only in microaerophilically and anaerobically cultured bacteria. Nitrite reductase was detected in cultures grown with  $91 \mu\text{M-O}_2$  but not with  $127 \mu\text{M-O}_2$ . For both enzymes maximum activity was only found under complete anaerobiosis.

Although nitrate and nitrite reductases were found in cultures held under low oxygen steady states, no nitrate reduction was detected in the cultures, indicating that oxygen is also inhibitory to nitrate reduction. Nitrate reductase was formed by anaerobic organisms growing in the absence of nitrate using nitrous oxide as oxidant, but it was virtually absent from nitrite limited cultures lacking nitrate, indicating repression of nitrate reductase synthesis in organisms using nitrite as oxidant. These findings contrast with the metabolically similar *Thiomicrospira denitrificans* (Timmer-ten Hoor, 1977) in which nitrate reductase is constitutive. The similar levels of nitrite reductase found in anaerobic nitrate and nitrite limited cultures suggested that this enzyme was fully derepressed under all the growth conditions tested. Also the growth of cell suspensions anaerobically with nitrate showed the production of nitrite that was rapidly taken up, as well as the reduction of nitrite in the nitrite suspensions.

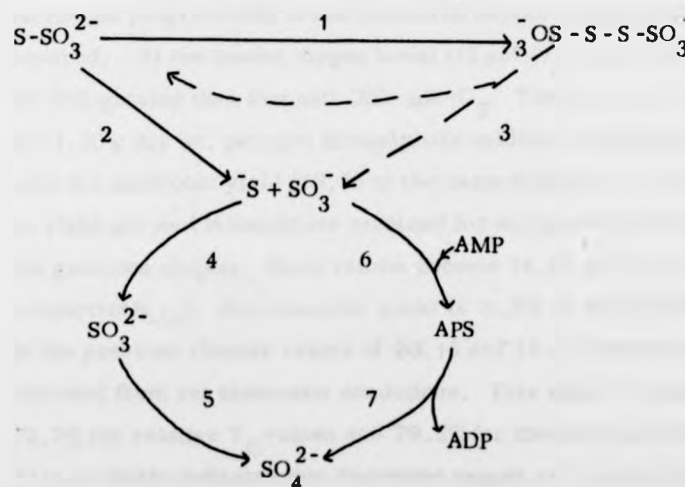
Nitrous oxide is believed to be the last intermediate before nitrogen in the nitrate reduction pathway, requiring nitrous-oxide reductase for its reduction to nitrogen (Aminuddin and Nicholas, 1973; Ishaque and Aleem, 1973; Baldensperger and Garcia, 1975). This enzyme is very labile and has not yet been characterized (Thauer, Jungermann and Decker, 1977). Attempts by Baldensperger and Garcia (1975) to detect this enzyme in T. denitrificans in crude extracts by methods described by Payne, Riley and Cox, 1971, proved unsuccessful. The activity of nitrate and nitrite reductase was found to be not as high as in other anaerobic cultures, thus suggesting that they were partially derepressed in the presence of nitrous oxide. The ability of the organism to increase its yield immediately when transferred from nitrous oxide to nitrate indicates the presence of nitrate and nitrite reductases.

The two enzymes possibly concerned in sulphur compound oxidation, thiosulphate- and sulphur-oxidizing enzymes, were present at relatively very low levels in aerobic T. denitrificans and were repressed by 70 to 90% during anaerobic growth. The activity of thiosulphate oxidising enzyme in anaerobic cultures was only 0.30% of that in some aerobic thiobacilli (Kelly, 1968; Silver and Lundgren, 1968b), but similar to that in aerobic Thiobacillus A2 (Kelly, 1973). Earlier, Trudinger (personal communication to D.P. Kelly) was unable to detect this enzyme in a different strain of T. denitrificans. The involvement of polythionates in the oxidation of sulphur compounds has been in dispute since its proposal by Tamiya, Haga and Huzisige (1941) and Vishniac (1952). Tetrathionate has been found to accumulate during the oxidation of thiosulphate by various thiobacilli grown under certain conditions (Vishniac and Santer, 1957; Trudinger, 1964). Aerobically grown T. denitrificans has been found to accumulate small amounts of tetrathionate when growing in batch or low dilution rates. This was rapidly used and none was found to accumulate during higher dilution rates. Hence the discovery of small amounts of thiosulphate-

oxidizing enzyme in aerobic cell extracts is not unexpected. Lyric and Suzuki (1970) put forward a theory suggesting that when thiosulphate is supplied as the energy source, it can be metabolized in two ways: (i) converted to tetrathionate by thiosulphate-oxidizing enzyme, or (ii) split to form sulphur and sulphide. The oxidation of thiosulphate to tetrathionate and the subsequent reductive cleavage of tetrathionate to thiosulphate, sulphur and sulphide resulted in no net gain of energy. The splitting of thiosulphate produces the same result but is only a one-step reaction, and is thought to be the first step in the pathway of thiosulphate oxidation to sulphate. The thiosulphate-oxidizing enzyme appeared to them to initiate a secondary pathway operative under conditions of high initial thiosulphate concentrations (Scheme 2). A similar phenomenon has been reported in heterotrophic bacteria (Trudinger, 1967), Pseudomonas fluorescens, Pseudomonas aeruginosa, Achromobacter stutzeri and Thiobacillus trautweinii. When these were supplied with high quantities of thiosulphate, enzymes were induced which were capable of oxidizing thiosulphate to tetrathionate.

The activity of the glutathione-dependent sulphur-oxidizing enzyme was at a maximum around 1  $\mu\text{mol}$  sulphur oxidized per mg protein in 210 min. This is very low activity, but is of the same order as found for T. thiooxidans, T. thioparus and T. ferrooxidans (Suzuki, 1965a, b; Suzuki and Silver, 1966; Silver and Lundgren, 1968a). The organism growing aerobically at a dilution rate of  $0.08 \text{ h}^{-1}$  oxidized thiosulphate at a rate of  $10.3 \mu\text{mol (g dry wt.)}^{-1} \text{ h}^{-1}$ . The protein content of aerobic bacteria was approximately 50% (w/w) of the dry weight, so the  $\text{qS}_2\text{O}_3$  was equivalent to  $20.5 \mu\text{mol (mg total protein)}^{-1} \text{ h}^{-1}$  or a rate some 80 times more rapid than could be accounted for by the sulphur-oxidizing enzyme oxidation of the sulphane-sulphur of thiosulphate. It must hence be concluded that these results do not support the view that the sulphur-oxidizing enzyme can be wholly involved in thiosulphate metabolism in T. denitrificans. Also there is no obvious role for the enzyme activity observed in the anaerobic bacteria, unless this enzyme had another, as yet undetected, function.

**SCHEME 2** Scheme for thiosulphate oxidation (after Lyric and Suzuki, 1970).



1 = Thiosulphate-oxidizing enzyme

2 = Thiosulphate splitting enzyme (rhodanese or reductase)

3 = Enzymes unknown

4 = Sulphur-oxidizing enzyme

5 = Sulphite oxidase (Aminuddin and Nicholas, 1974)

6 = APS reductase (Aminuddin and Nicholas, 1974)

7 = ADP sulphyrylase (Aminuddin and Nicholas, 1974)

T. denitrificans can be switched back and forth in the chemostat between aerobic and anaerobic growth with no permanent loss of nitrate-reducing capacity during aerobiosis. It appears, however, that oxygen is an inhibitory substrate as aerobic growth yields increased progressively as the dissolved oxygen concentration was lowered. At the lowest oxygen level ( $12 \mu\text{M-O}_2$ ) the yield was 60-70% greater than that with  $200 \mu\text{M-O}_2$ . The yield at  $12 \mu\text{M-O}_2$  of 11.50 g dry wt. per mol thiosulphate oxidized should be compared with the anaerobic yield of 9.33 at the same dilution rate. Calculated as yield per mol thiosulphate oxidized for energetic purposes as in the previous chapter, these values become 14.88 and 11.44 respectively, i.e. the anaerobic yield is 76.9% of the aerobic yield. In the previous chapter values of 20.18 and 14.67 respectively were obtained from set chemostat conditions. This may be compared with 72.7% for relative  $Y_G$  values and 79.2% for theoretical available energy. This probably indicates that dissolved oxygen at  $12 \mu\text{M}$  is optimal for the efficient aerobic growth of T. denitrificans. While T. denitrificans grows best at low dissolved oxygen concentrations, it is still capable of producing greater growth yields at high dissolved oxygen than obligately aerobic thiobacilli mentioned in the previous chapter. This is probably due to a more efficient energy conserving sulphur oxidation mechanism.

PART 5: ATTEMPTS TO ELUCIDATE BIOENERGETICS  
USING NON-GROWING CELL SUSPENSIONS

## PART 5: ATTEMPTS TO ELUCIDATE BIOENERGETICS USING NON-GROWING CELL SUSPENSIONS

### 5.1 Introduction

The chemostat studies indicated that the amount of growth coupled to substrate use was constant for the given conditions of reductant and oxidant limitation. The oxidation of thiosulphate is coupled to carbon dioxide fixation which requires a supply of reducing power in the form of NADH and energy as ATP. The chemostat data showed aerobic growth to be greater than anaerobic growth in agreement with the oxidation thermodynamics. The Y<sub>g</sub> calculations predicted that 6-7 ATP are formed from one mole thiosulphate aerobically and 4-5 anaerobically. Two types of phosphorylation have been reported in T. denitrificans: substrate level phosphorylation where sulphite combines with AMP to form adenosine phosphosulphate (APS) and finally sulphate and ADP (Bowen et al., 1966; Sargeant et al., 1966; Aminuddin and Nicholas, 1974a); oxidative phosphorylation where sulphite is oxidized to sulphate via sulphite oxidase and the electron transport chain with nitrate or oxygen as terminal oxidants (Aminuddin and Nicholas, 1974b).

Earlier work with the aerobic T. neapolitanus demonstrated that one way to obtain information about the occurrence of oxidative phosphorylation and the relative contribution of oxidative and substrate-level phosphorylation was to attempt selective inhibition of the former with uncoupling agents such as 2:4-dinitrophenol (Kelly and Syrett, 1963, 1964, 1966a). A consequence of inhibiting ATP synthesis with DNP would be the inhibition of carbon dioxide fixation (Kelly and Syrett, 1963), but DNP could also inhibit carbon dioxide fixation in consequence of it preventing NAD<sup>+</sup> reduction, which is effected by energy-dependent electron flow from cytochrome c (or b) and would be sensitive to uncoupling agents (Kelly and Syrett, 1966a; Kelly, 1978).

An attempt was made to apply the uncoupling experiment methods to aerobic and anaerobic suspensions of T. denitrificans oxidizing thiosulphate or sulphide and fixing carbon dioxide. The rationale was to seek to demonstrate the occurrence of both substrate-level and oxidative phosphorylation and to attempt to quantitate their relative contribution to energy conservation under aerobic and anaerobic conditions. If the difference in growth yield observed between aerobic T. denitrificans and other aerobic thiobacilli is due to a greater extent of oxidative phosphorylation in the former (see Part 3), then carbon dioxide fixation during both sulphide and thiosulphate oxidation might be expected to be more sensitive to DNP than in aerobic thiobacilli, and for less difference in inhibition with these two substrates to be observed than with T. neapolitanus.

## 5.2 Suspensions of T. denitrificans oxidizing thiosulphate, sulphur and sulphide

### 5.2.1 Anaerobic cell suspensions

Cell suspensions in the absence of any sulphur compound but in the presence of nitrate evolved  $0.87 \mu\text{mol gas (mg protein)}^{-1}$  (mean value) and fixed  $2,233 \text{ cpm } ^{14}\text{CO}_2 \text{ (mg protein)}^{-1}$ . The amount of gas evolved and  $^{14}\text{CO}_2$  fixed increased in proportion to the thiosulphate and sulphide concentrations. The cell suspensions decreased in  $^{14}\text{CO}_2$  fixation and evolution of gas with increase in cell density (Table 18). The gas evolved by the thin cell suspensions was similar to the theoretical, i.e. the latter values for nitrogen evolution from 3, 6, 9  $\mu\text{mole thiosulphate}$  are 2.4, 4.8 and 7.2  $\mu\text{mole}$  with the experimental values of 2.37, 4.45 and 6.43 respectively (after correcting for values obtained in the absence of thiosulphate).

In the presence of sulphur, low levels of  $^{14}\text{CO}_2$  were fixed in proportion to the cell suspensions, whereas with sulphide the cell suspensions fixed more  $^{14}\text{CO}_2$  which was in proportion to the sulphide concentration. In both cases the evolution of the gas was irregular (Table 19).



TABLE 18 Comparison of  $^{14}\text{CO}_2$  fixation and gas release from anaerobic cell suspensions grown on thiosulphate and nitrate limited medium

Growth limiting nutrient	Organism concentration (mg protein)	$\text{KNO}_3$ ( $\mu\text{mole}$ )	$\text{Na}_2\text{S}_2\text{O}_3$ ( $\mu\text{mole}$ )	Gas evolved ( $\mu\text{mole}$ )	$^{14}\text{CO}_2$ fixation (cpm)*	Gas evolved per $\mu\text{mole}$ $\text{Na}_2\text{S}_2\text{O}_3$	$^{14}\text{CO}_2$ fixation per mole $\text{Na}_2\text{S}_2\text{O}_3$ (cpm)*	Incubation time (min)
Thiosulphate	6.0	12	0	5.74	13,127	-	-	100
	6.0	6	3	8.11	208,449	2.70	69,483	100
	6.0	12	6	10.19	369,974	1.70	61,662	100
	6.0	18	9	12.17	572,116	1.35	63,568	100
	12.1	12	0	-	25,009	-	-	100
	12.1	6	3	3.75	191,130	1.25	63,710	100
	12.1	12	6	4.25	340,250	0.71	56,708	100
	12.1	18	9	13.95	595,359	1.55	66,151	100
	8.4	10	0	6.66	19,313	-	-	120
	4.2	10	10	7.66	472,177	0.77	47,218	120
Nitrate	8.4	10	10	8.93	492,035	0.89	49,204	120
	16.8	10	10	-	378,233	-	37,823	120

\* 355, 200 cpm  $\equiv$  1  $\mu\text{mol CO}_2$

TABLE 19 Comparison of  $^{14}\text{CO}_2$  fixation and gas release from anaerobic cell suspensions grown on sulphur and sulphide

Growth limiting nutrient	Organism concentration (mg protein)	$\text{KNO}_3$ ( $\mu\text{mole}$ )	$\text{Na}_2\text{S}$ ( $\mu\text{mole}$ )	S ( $\text{mg}/2$ )	Gas evolved ( $\mu\text{mole}$ )	$^{14}\text{CO}_2$ fixation (cpm) *1	Gas evolved per $\mu\text{mole Na}_2\text{S}$ or mg S ( $\mu\text{mole}$ )	$^{14}\text{CO}_2$ fixation per $\mu\text{mole Na}_2\text{S}$ or mg S ( $\mu\text{mole}$ ) *1	Incubation time (mins)
Sulphide	3.7	8	0	-	5.33	7,797	-	-	330
	3.7	4	2	-	2.53	52,813	1.27	26,407	330
	3.7	8	4	-	4.89	135,043	1.22	33,761	330
	3.7	12	6	-	4.94	183,989	0.82	31,665	330
	7.4	8	0	-	5.97	11,351	-	-	330
	7.4	4	2	-	4.50	57,743	2.25	28,872	330
	7.4	8	4	-	5.92	113,540	1.48	28,385	330
	7.4	12	6	-	5.94	144,962	0.99	24,160	330
Nitrate	8.42	-	-	0	-	17,126	-	-	120
	4.21	-	-	10	1.06	20,928	0.11	2,093	120
	8.42	-	-	10	0.70	25,085	0.07	2,509	120
	16.84	-	-	10	11.07	30,736	1.11	3,074	120

\*1 355,200 cpm  $\equiv$  1  $\mu\text{mol CO}_2$ \*2 Sulphur from 5 day old *T. thiooxidans* culture

From this preliminary investigation, future work was based on thin cell suspensions of approximately 6 mg protein; 6  $\mu$ mole thiosulphate and 12  $\mu$ mole nitrate in thiosulphate-limited suspensions; 10  $\mu$ mole thiosulphate and 10  $\mu$ mole nitrate in nitrate-limited suspensions; 4  $\mu$ mole sulphide and 8  $\mu$ mole nitrate in sulphide suspensions (all in a total volume of 2.5 ml).

#### 5.22 Aerobic cell suspensions

In the absence of any sulphur compound, the cell suspensions fixed 12,641 cpm  $^{14}\text{CO}_2$  (mg protein) $^{-1}$  and consumed 3.72  $\mu$ mole oxygen (mg protein) $^{-1}$ . Both dense and thin suspensions resulted in an increase of  $^{14}\text{CO}_2$  fixed in proportion with the thiosulphate and sulphide concentration.

In the presence of sulphur, the  $^{14}\text{CO}_2$  fixed was the same as that in its absence, i.e. 1,892 and 1,683 cpm  $^{14}\text{CO}_2$  (mg protein) $^{-1}$ , respectively. The comparison of the amount of  $^{14}\text{CO}_2$  fixed between the two different suspensions, in thiosulphate and sulphide, resulted in the thin suspensions fixing  $^{14}\text{CO}_2$  three to five, and two to three times more, respectively, than the dense suspensions (Table 20).

In an attempt to eliminate the activity of the cells in the absence of any sulphur compound, the cells were starved for 15 h. This resulted in increased  $^{14}\text{CO}_2$  fixation with thiosulphate and sulphide, but did not remove the background activity of 2,332 and 2,119 cpm (mg protein) $^{-1}$  respectively (Table 21).

TABLE 20 Comparison of  $^{14}\text{CO}_2$  fixation and oxygen uptake from aerobic cell suspensions grown on thiosulphate and sulphide

Growth limiting nutrient	Organism concentration (mg protein)	$\text{Na}_2\text{S}_2\text{O}_3$ ( $\mu\text{mole}$ )	$\text{Na}_2\text{S}$ ( $\mu\text{mole}$ )	Gas uptake ( $\mu\text{mole}$ )	$^{14}\text{CO}_2$ fixation (cpm) *	Gas uptake per $\mu\text{mole}$ $\text{Na}_2\text{S}$ or $\text{Na}_2\text{S}_2\text{O}_3$ ( $\mu\text{mole}$ )	$^{14}\text{CO}_2$ fixation per $\mu\text{mole}$ $\text{Na}_2\text{S}$ or $\text{Na}_2\text{S}_2\text{O}_3$ ( $\mu\text{mole}$ )	Incubation time (mins)
Thiosulphate	5.5	0	-	20.46	69,528	-	-	243
	5.5	3	-	25.57	231,164	8.52	77,055	243
	5.5	6	-	28.21	298,021	4.70	49,670	243
	5.5	9	-	30.81	403,688	3.42	44,854	243
	11.0	0	-	-	54,765	-	-	243
	11.0	3	-	-	128,502	-	42,834	243
	11.0	6	-	36.47	158,210	6.08	26,368	243
	11.0	9	-	32.58	266,806	3.62	29,645	243
	4.75	-	0	5.92	31,005	-	-	180
	4.75	-	2	6.40	95,531	3.2	47,766	180
Sulphide	4.75	-	4	11.54	105,159	2.89	26,290	180
	4.75	-	6	12.74	152,635	2.12	25,439	180
	9.5	-	0	14.20	50,432	-	-	180
	9.5	-	2	17.52	81,914	8.76	40,957	180
	9.5	-	4	19.56	94,247	4.89	23,562	180
	9.5	-	6	23.10	97,718	3.85	16,286	180

\* 355,200 cpm  $\equiv$  1  $\mu\text{mol CO}_2$

This preliminary investigation into the best conditions for future experimental work resulted in thin cell suspensions, 6  $\mu$ mole thiosulphate and 4  $\mu$ mole sulphide being used in a total volume of 2.5 ml.

### 5.3 The effect of an uncoupling agent on carbon dioxide fixation of *T. denitrificans*

2,4-dinitrophenol was used as the uncoupling agent in this investigation due to its precise reaction in inhibiting oxidative phosphorylation and not substrate-level phosphorylation, though it can also prevent the  $\text{NAD}^+$  reduction dependent on energy-dependent "reversed electron flow" and thus affect the assimilation of carbon dioxide.

#### 5.3.1 The effect of 2,4-dinitrophenol on carbon dioxide fixation under anaerobic conditions with thiosulphate

In the absence of thiosulphate, suspensions fixed  $^{14}\text{CO}_2$  in relation to the dinitrophenol (DNP) concentration, i.e.,  $^{14}\text{CO}_2$  fixation at  $10^{-4}$  M DNP was stimulated from 2,787 cpm (mg protein) $^{-1}$  with no dinitrophenol to 3,121 cpm (mg protein) $^{-1}$ .  $10^{-5}$  M DNP did not stimulate activity, whereas  $10^{-3}$  M DNP inhibited  $^{14}\text{CO}_2$  fixation. In the presence of 6  $\mu$ mole thiosulphate in a thiosulphate-limited suspensions,  $^{14}\text{CO}_2$  fixation was stimulated at  $10^{-4}$  M and  $10^{-5}$  M DNP.  $10^{-3}$  M DNP had the same inhibitory effect as in the absence of thiosulphate (Table 22). In the absence of thiosulphate, the manometric readings gave results in the same relationship as for  $^{14}\text{CO}_2$  fixation. There was no significant difference in the gas exchange in the presence of thiosulphate. Fig. 29 demonstrates the effect of DNP on the rate of gas evolution and  $^{14}\text{CO}_2$  fixation by thin cell suspensions.

The oxidation of thiosulphate was affected by DNP, resulting in tetrathionate production at 0,  $10^{-5}$  and  $10^{-4}$  M DNP of 0.64, 1.38 and 1.38  $\mu$ mole respectively. At  $10^{-3}$  M DNP, 1.38  $\mu$ mole thiosulphate remained and 1.84  $\mu$ mole tetrathionate was produced. No trithionate accumulated. The nitrate-limited suspensions seemed to accumulate

TABLE 21 Comparison of  $^{14}\text{CO}_2$  fixation and oxygen uptake from thin cell suspensions, starved and unstarved, grown on thiosulphate and sulphide

Growth limiting nutrient	Organism concentration (mg protein)	$\text{Na}_2\text{S}_2\text{O}_3$ ( $\mu\text{mole}$ )	$\text{Na}_2\text{S}$ ( $\mu\text{mole}$ )	Gas uptake ( $\mu\text{mole}$ )	$^{14}\text{CO}_2$ fixation (cpm) *	Corrected gas uptake ( $\mu\text{mole}$ )	Corrected $^{14}\text{CO}_2$ fixation (cpm) *	Incubation time (mins)
Thiosulphate	Unstarved	0	-	20.46	69,528	-	-	243
	(5.5)	6	-	28.21	298,021	7.75	228,493	243
	Starved	0	-	0.33	7,811	-	-	270
	(3.35)	6	-	7.86	314,091	7.53	306,280	270
Sulphide	Unstarved	-	0	5.92	31,005	-	-	180
	(5.5)	-	4	11.54	105,159	5.62	74,154	180
	Starved	-	0	-0.25	7,100	-	-	270
	(3.35)	-	4	5.22	148,188	5.47	141,088	270

\* 355,200 cpm  $\equiv$  1  $\mu\text{mol CO}_2$



TABLE 22 The effect of 2:4-dinitrophenol on  $^{14}\text{CO}_2$  fixation and gas release coupled to thiosulphate oxidation anaerobically

Growth limiting nutrient	by thin cell suspensions				Residual polythionates $\text{S}_4\text{O}_6^{2-}$ - $\text{S}_2\text{O}_3^{2-}$ - $\text{S}_3\text{O}_6^{2-}$ ( $\mu\text{mole}$ ) ( $\mu\text{mole}$ ) ( $\mu\text{mole}$ )	Gas evolved ( $\mu\text{mole}$ )	$^{14}\text{CO}_2$ fixation (cpm) *	Gas uptake per $\mu\text{mole}$ $\text{Na}_2\text{S}_2\text{O}_3$ ( $\mu\text{mole}$ )	$^{14}\text{CO}_2$ fixation per $\mu\text{mole}$ $\text{Na}_2\text{S}_2\text{O}_3$ ( $\mu\text{mole}$ )	Incubation time (mins)	Organism conc. (mg protein)
	DNP (M)	$\text{KNO}_3$ ( $\mu\text{mole}$ )	$\text{Na}_2\text{S}_2\text{O}_3$ ( $\mu\text{mole}$ )								
Thiosulphate	0	12	0	-	-	10.35	12,692	-	-	300	4.65
	$10^{-5}$	12	0	-	-	9.48	12,123	-	-		
	$10^{-4}$	12	0	-	-	8.48	14,514	-	-		
	$10^{-3}$	12	0	-	-	3.76	5,092	-	-		
	0	12	6	0.64	0	10.59	176,040	1.77	29,340		
	$10^{-5}$	12	6	1.38	0	9.04	199,334	1.51	33,222		
	$10^{-4}$	12	6	1.38	0	13.10	230,112	2.18	38,352		
	$10^{-3}$	12	6	1.84	1.38	4.12	8,766	0.69	1,461		
	0	10	0	-	-	11.83	23,467	-	-		
	$10^{-5}$	10	0	-	-	10.46	15,180	-	-		
Nitrate	$10^{-4}$	10	0	-	-	10.38	21,883	-	-	240	8.05
	$10^{-3}$	10	0	-	-	5.08	12,548	-	-		
	0	10	10	7.08	0	9.77	448,269	0.98	44,827		
	$10^{-5}$	10	10	8.92	0	8.66	475,503	0.87	47,550		
	$10^{-4}$	10	10	9.84	0	9.21	275,655	0.92	27,566		
	$10^{-3}$	10	10	9.84	0	4.87	13,500	0.49	1,350		

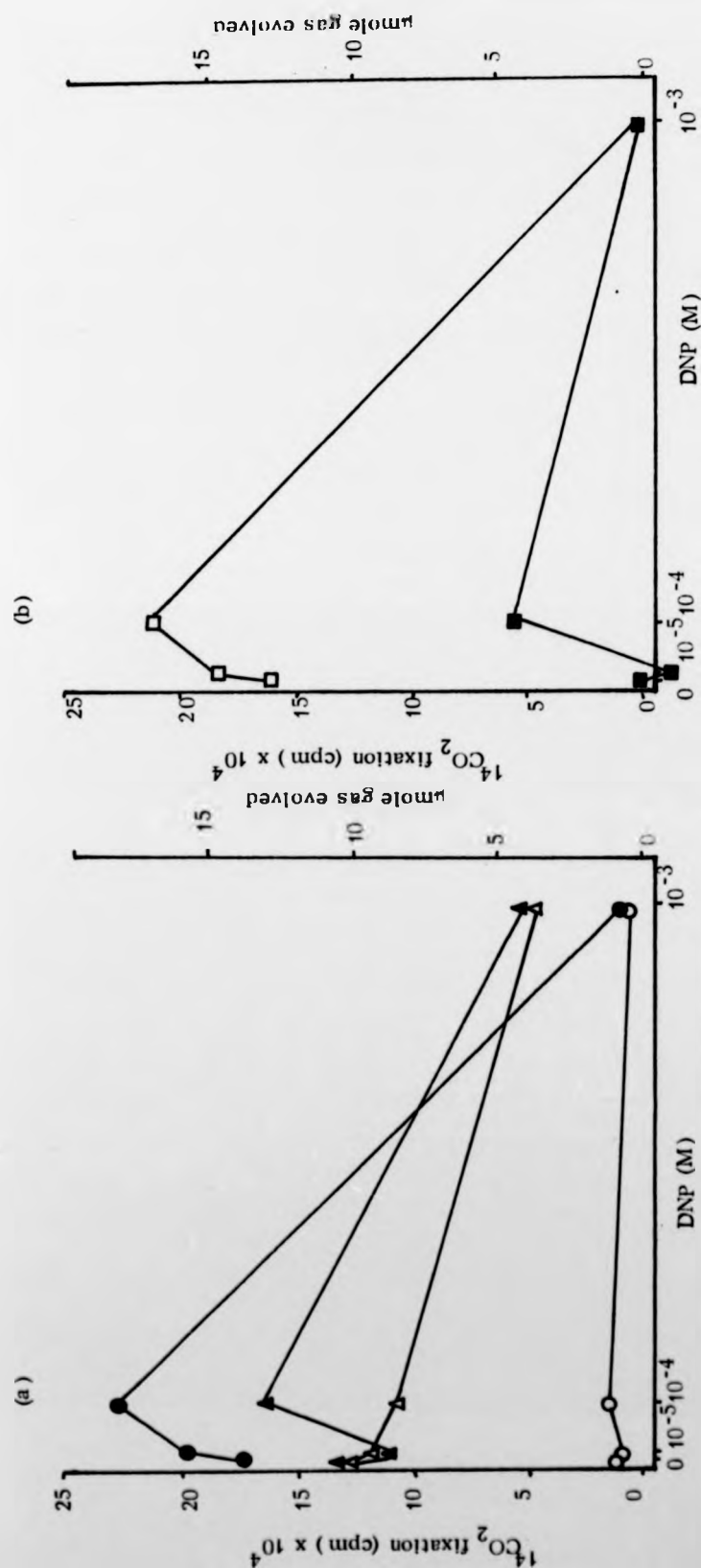
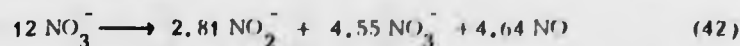


FIG. 29 Effect of 2,4-dinitrophenol on  $^{14}\text{CO}_2$  fixation and gas release coupled to anaerobic thiosulphate oxidation by thin thiosulphate limited cell suspensions. (a)  $^{14}\text{CO}_2$  fixation without  $\text{Na}_2\text{S}_2\text{O}_3$  (O), with 6  $\mu\text{mole Na}_2\text{S}_2\text{O}_3$  (●); gas released without  $\text{Na}_2\text{S}_2\text{O}_3$  ( $\Delta$ ), with 6  $\mu\text{mole Na}_2\text{S}_2\text{O}_3$  (■). (b) Gas release (■),  $^{14}\text{CO}_2$  fixation (□), corrected for reaction in absence of  $\text{Na}_2\text{S}_2\text{O}_3$ . Cell suspensions = 4.65 mg protein.



tetrathionate more than could be possibly produced from the thiosulphate. Any sulphur or sulphide present would increase the tetrathionate colour of the assay, hence give an apparent high tetrathionate value. As the initial readings for thiosulphate and tetrathionate were the same, then the tetrathionate colour was probably due to sulphide or sulphur.

In a further investigation using  $10^{-4}$  M DNP only, the nitrate and nitrite content enabled the calculation of the overall reaction effected by the cells (Table 23). In flasks with 12  $\mu$ mole nitrate, only 4.66  $\mu$ mole gas was evolved, 2.81  $\mu$ mole nitrite was found to accumulate and 4.55  $\mu$ mole nitrate remained. According to equation 31, 12  $\mu$ mole nitrate should be reduced to 6  $\mu$ mole nitrogen, in the presence of thiosulphate. In its absence, 4.46  $\mu$ mole nitrate was converted to a nitrogenous gas  $[12 - (2.81 + 4.55)]$ . If this was reduced to nitrogen only 2.32  $\mu$ mole would be recorded, but if it was reduced to nitric oxide only, then 4.66  $\mu$ mole would be evolved, which is very close to that recorded. Hence it is assumed that the following occurred:

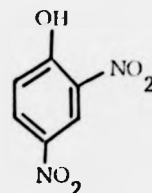


The  $^{14}\text{CO}_2$  fixation with  $10^{-4}$  M DNP was slightly inhibited, with the complete oxidation of thiosulphate and accumulation of 2.55  $\mu$ mole nitrite and 4.55  $\mu$ mole nitrate remaining, and thus 4.9  $\mu$ mole nitrate was reduced further (Table 23). 8.41  $\mu$ mole gaseous product was evolved. If the gaseous result in the absence of thiosulphate is taken into account, then 4.38  $\mu$ mole gas was evolved which would be close to 4.9  $\mu$ mole nitrous oxide. The amount of gas evolved from the cells in  $10^{-4}$  M DNP with no thiosulphate is much higher than the possible release of nitric oxide, nitrous oxide or nitrogen, i.e., 4.03  $\mu$ mole recorded but only 2.83  $\mu$ mole nitrate could be reduced. The composition of dinitrophenol includes two  $-\text{NO}_2$  groups.

TABLE 23 The effect of  $10^{-4}$  M 2,4-dinitrophenol on  $^{14}\text{CO}_2$  fixation and nitrate reduction coupled to anaerobic thiosulphate and sulphide oxidation by thin cell suspensions

Growth limiting nutrient	DNP (M)	$\text{KNO}_3$ ( $\mu\text{mole}$ )	$\text{Na}_2\text{S}_2\text{O}_3$ ( $\mu\text{mole}$ )	$\text{Na}_2\text{S}$ ( $\mu\text{mole}$ )	End products ( $\mu\text{mole}$ )					Gas evolved ( $\mu\text{mole}$ )	$^{14}\text{CO}_2$ evolved fixation (cpm)	Gas evolved per $\mu\text{mole}$ $\text{Na}_2\text{S}_2\text{O}_3$ or $\text{Na}_2\text{S}$ ( $\mu\text{mole}$ )	$^{14}\text{CO}_2$ fixation per $\mu\text{mole}$ $\text{Na}_2\text{S}_2\text{O}_3$ or $\text{Na}_2\text{S}$ (cpm)
					$\text{SO}_4^{2-}$	$\text{S}_4\text{O}_6^{2-}$	$\text{S}_2\text{O}_3^{2-}$	$\text{S}_3\text{O}_6^{2-}$	$\text{NO}_3^-$				
Thiosulphate	$10^{-4}$	12	6	-	16.77	0	0	0	4.55	2.55	8.41	214,283	1.40
210 mins	$10^{-4}$	12	0	-	-	-	-	-	5.93	3.24	4.03	7,700	-
5.5 mg protein	0	12	6	-	16.07	0.09	0.51	0	3.17	3.63	7.79	283,338	1.30
	0	12	0	-	-	-	-	-	4.55	2.81	4.66	16,601	-
Sulphide	$10^{-4}$	8	-	4	3.91	-	-	-	0	1.196	2.86	90,742	0.72
210 mins	$10^{-4}$	8	-	0	-	-	-	-	0.89	3.496	3.07	3,071	-
5.45 mg protein	0	8	-	4	2.03	-	-	-	0	1.656	4.73	113,108	1.18
	0	8	-	0	-	-	-	-	2.28	2.53	4.38	6,351	-

\* 355, 200 cpm  $\equiv$  1  $\mu\text{mole CO}_2$



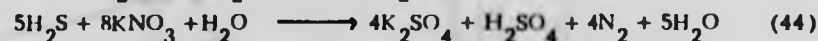
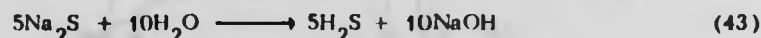
If T. denitrificans could attack this molecule and reduce these groups to nitrogen, the overall gaseous output would rise.

The anaerobic nitrate-limited cell suspensions evolved more gas in the absence of thiosulphate than in its presence at all DNP values. DNP inhibited  $^{14}\text{CO}_2$  fixation at all concentrations whether thiosulphate was present or not, except at  $10^{-5}$  M DNP which stimulated  $^{14}\text{CO}_2$  fixation in the presence of thiosulphate (Table 22). Fig. 30 demonstrates this effect of DNP on the rate of gas evolved, and  $^{14}\text{CO}_2$  fixation by thin cell suspensions oxidizing thiosulphate.

### 5.3.2 The effect of 2,4-dinitrophenol on carbon dioxide fixation under anaerobic conditions with sulphide

The anaerobic cell suspensions evolved more gas without sulphide than with (Table 24), whereas the  $^{14}\text{CO}_2$  fixed is greater in those cells with sulphide than those without, i.e. 12,744 and 2,716 cpm (mg protein) $^{-1}$  respectively. The most  $^{14}\text{CO}_2$  fixed was with cells suspended with  $10^{-5}$  M DNP and without sulphide. Activity at  $10^{-3}$  M DNP was similar to that without DNP, in the absence of sulphide, whereas with sulphide, the  $^{14}\text{CO}_2$  fixation was inhibited. Fig. 31 demonstrates this effect of DNP on the rate of gas evolved and  $^{14}\text{CO}_2$  fixed by thin cell suspensions oxidizing sulphide.

At  $10^{-4}$  M DNP the nitrate and nitrite content enabled the calculation of the final reaction of the cells (Table 23). In the absence of both dinitrophenol and sulphide, but in the presence of 8  $\mu\text{mole}$  nitrate 4.38  $\mu\text{mole}$  gas was evolved, 2.53  $\mu\text{mole}$  nitrite accumulated and 2.28  $\mu\text{mole}$  nitrate remained.



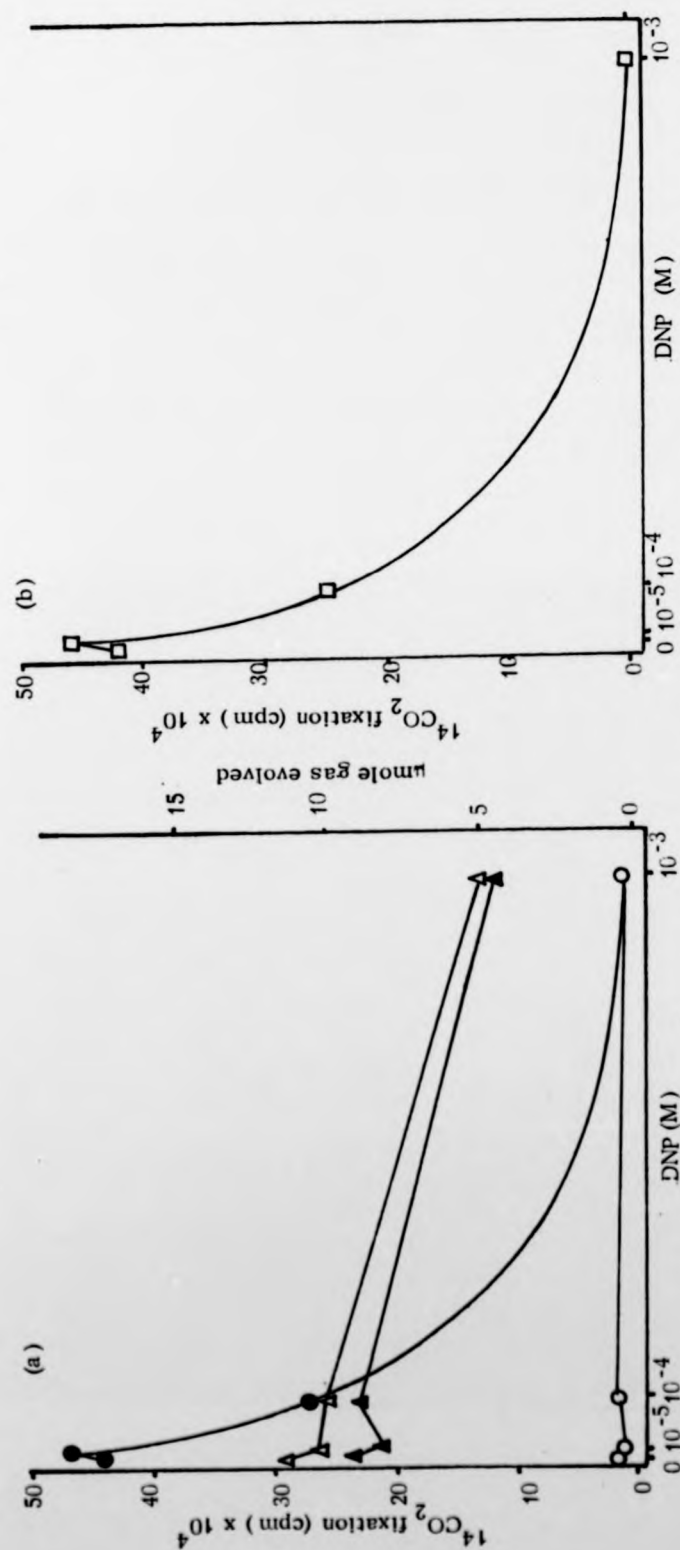


FIG. 30 Effect of 2:4-dinitrophenol on  $^{14}\text{CO}_2$  fixation and gas release coupled to anaerobic thiosulphate oxidation by thin, nitrate limited cell suspensions. (a)  $^{14}\text{CO}_2$  fixation without  $\text{Na}_2\text{S}_2\text{O}_3$  (O), with 10  $\mu\text{mole Na}_2\text{S}_2\text{O}_3$  (●); gas released without  $\text{Na}_2\text{S}_2\text{O}_3$  (Δ), with 10  $\mu\text{mole Na}_2\text{S}_2\text{O}_3$  (▲). (b)  $^{14}\text{CO}_2$  fixation (□) corrected for reaction in absence of  $\text{Na}_2\text{S}_2\text{O}_3$ . Cell suspensions = 8.05 mg protein.

TABLE 24 The effect of 2:4-dinitrophenol on  $^{14}\text{CO}_2$  fixation and gas release coupled to anaerobic sulphide oxidation by thin cell suspensions

Growth limiting nutrient	DNP (M)	$\text{KNO}_3$ ( $\mu\text{mole}$ )	$\text{Na}_2\text{S}$ ( $\mu\text{mole}$ )	Gas evolved ( $\mu\text{mole}$ )	$^{14}\text{CO}_2$ fixation (cpm) *	Gas evolved per $\mu\text{mole Na}_2\text{S}$ ( $\mu\text{mole}$ )	$^{14}\text{CO}_2$ fixation per $\mu\text{mole Na}_2\text{S}$ (cpm) *	Incubation time (mins)	Organism concentration (mg protein)
Sulphide	0	8	0	9.64	17,516	-	-	300	6.45
	$10^{-5}$	8	0	9.53	24,733	-	-		
	$10^{-4}$	8	0	8.85	24,298	-	-		
	$10^{-3}$	8	0	3.49	14,952	-	-		
	0	8	4	5.92	80,247	1.48	20,062		
	$10^{-5}$	8	4	5.74	103,124	1.44	25,781		
	$10^{-4}$	8	4	4.99	99,205	1.25	24,801		
	$10^{-3}$	8	4	5.99	15,818	1.50	3,955		

\* 355,200 cpm  $\equiv$  1  $\mu\text{mole CO}_2$

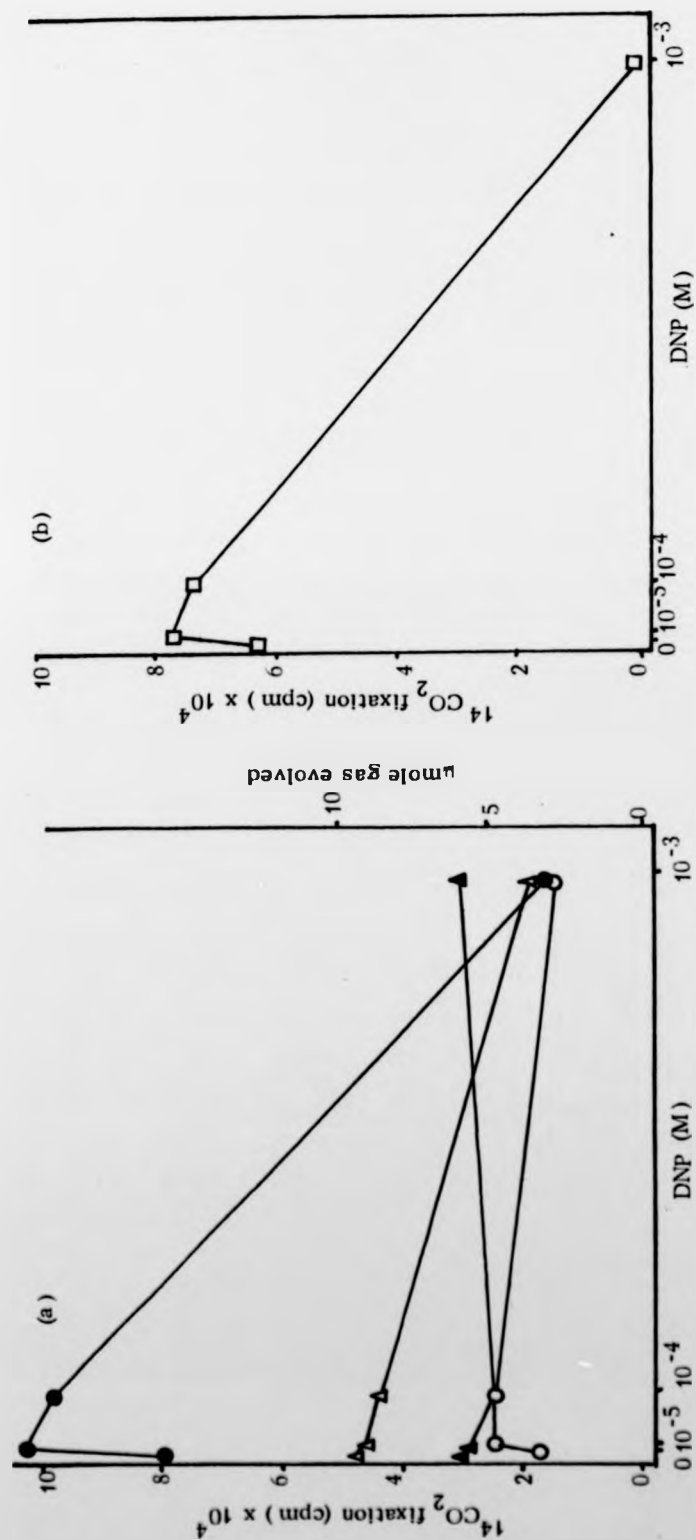


FIG. 31 Effect of 2,4-dinitrophenol on  $^{14}\text{CO}_2$  fixation and gas release coupled to anaerobic sulphide oxidation by thin sulphide limited cell suspensions. (a)  $^{14}\text{CO}_2$  fixation without  $\text{Na}_2\text{S}$  (O), with 4  $\mu\text{mole Na}_2\text{S}$  (●); gas release without  $\text{Na}_2\text{S}$  ( $\Delta$ ), with 4  $\mu\text{mole Na}_2\text{S}$  ( $\blacktriangle$ ). (b)  $^{14}\text{CO}_2$  fixation ( $\square$ ) corrected for reaction in absence of  $\text{Na}_2\text{S}$ . Cell suspensions = 6.45 mg protein.

Therefore,  $3.19 \mu\text{mole}$  nitrate  $[8 - (2.53 + 2.28)]$  was reduced to either  $3.19 \mu\text{mole}$  nitric oxide or  $1.6 \mu\text{mole}$  nitrogenous oxide or nitrogen. Thus the exact nature of the gas evolved could not be predicted. In the flask with sulphide but no DNP  $2.03 \mu\text{mole}$  sulphate was produced, indicating that only  $2.03 \mu\text{mole}$  sulphide was oxidized, which would only require  $3.24 \mu\text{mole}$  nitrate. All the nitrate was reduced with  $1.656 \mu\text{mole}$  nitrite remaining, hence  $6.344 \mu\text{mole}$  nitrate was reduced further. From the previous results it has been demonstrated how nitrate was reduced to other products in the absence of sulphide. So, if  $3.24 \mu\text{mole}$  nitrate was used during sulphide oxidation to form nitric oxide, therefore  $3.1 \mu\text{mole}$  nitrate remains to be reduced either to  $3.1 \mu\text{mole}$  nitric oxide or to  $1.55 \mu\text{mole}$  nitrous oxide or nitrogen. If it was reduced to the latter, then the total gas evolved would be  $4.78 \mu\text{mole}$ , which is in close agreement to the reading obtained of  $4.73 \mu\text{mole}$ . In the presence of both sulphide and  $10^{-4}$  M DNP, no nitrate remains, but  $1.196 \mu\text{mole}$  nitrite accumulates. By using the same idea as before, if  $3.91 \mu\text{mole}$  sulphate was produced, hence  $3.91 \mu\text{mole}$  sulphide was oxidized. This requires  $6.25 \mu\text{mole}$  nitrate.  $6.804 \mu\text{mole}$  nitrate can be further reduced. If out of this  $6.25 \mu\text{mole}$  nitrate is reduced to  $3.125 \mu\text{mole}$  nitrogen, this leaves  $0.554 \mu\text{mole}$  nitrate unaccounted for. So, as with thiosulphate, the nature of the gas evolved from the reaction with DNP is not easily derived. In the presence of  $10^{-4}$  M DNP and no sulphide,  $3.07 \mu\text{mole}$  gas was evolved which is probably nitric oxide, as  $3.496 \mu\text{mole}$  nitrite was produced and  $0.89 \mu\text{mole}$  nitrate remained, hence  $3.614 \mu\text{mole}$  nitrate was reduced further to either  $3.614 \mu\text{mole}$  nitric oxide or  $1.807 \mu\text{mole}$  nitrous oxide or nitrogen.

A test flask set up with no di-nitrophenol, thiosulphate, sulphide nor nitrate gave a final manometric result of  $0.49 \mu\text{mole}$  gas evolved  $(\text{mg protein})^{-1}$  and  $1,583 \text{ cpm } ^{14}\text{CO}_2$  fixed  $(\text{mg protein})^{-1}$ .



### 5.3.3 The effect of 2, 4-dinitrophenol on carbon dioxide fixation under aerobic conditions with thiosulphate

The cell suspensions in the absence of thiosulphate reacted as before. DNP was inhibitory at  $10^{-5}$ ,  $10^{-4}$  and  $10^{-3}$  M with and without thiosulphate (Table 25). Under anaerobic conditions, it was not possible to obtain a satisfactory result by subtracting those obtained without a sulphur compound present from those with a sulphur compound. Aerobically this led to a significant drop of  $^{14}\text{CO}_2$  fixation and oxygen uptake from no DNP to  $10^{-3}$  M DNP (Fig. 32).

4.92  $\mu\text{mole}$  thiosulphate was oxidized with 0.64  $\mu\text{mole}$  tetrathionate accumulating; excess sulphate was recorded due to its presence in the washed cell suspensions, both with and without  $10^{-4}$  M DNP (Table 26).

Starved cells with no thiosulphate still demonstrated oxygen uptake and  $^{14}\text{CO}_2$  fixation with  $10^{-4}$  M DNP, though much reduced as compared to unstarved cells (Table 27).

### 5.3.4 The effect of 2, 4-dinitrophenol on carbon dioxide fixation under aerobic conditions with sulphide.

$^{14}\text{CO}_2$  fixation in the absence of sulphide was relatively high compared to other conditions, i.e. 19,180 cpm (mg protein) $^{-1}$ , with no DNP. The  $^{14}\text{CO}_2$  fixation was inhibited at all concentrations of DNP in the absence of sulphide, whereas in its presence only  $10^{-4}$  and  $10^{-3}$  M DNP was inhibitory, and in both cases it was at these concentrations that oxygen uptake was at its greatest (Fig. 33). The action of DNP as an uncoupler of oxidative phosphorylation is supposed to be demonstrated by this reaction, where oxidation proceeds at a faster rate, yet no phosphorylation ensues, hence less  $^{14}\text{CO}_2$  is fixed (Table 25). Starved cells in the presence of sulphide and  $10^{-4}$  M DNP reacted in a similar manner as this, but at a higher overall activity as compared with unstarved cells. Aminuddin and Nicholas (1973) reported continued oxygen uptake in T. denitrificans after all the sulphide had been oxidized, due to the products being oxidized by the enzymes.



TABLE 25 The effect of 2,4-dinitrophenol on  $^{14}\text{CO}_2$  fixation and oxygen uptake coupled to aerobic thiosulphate and sulphide oxidation by thin cell suspensions

Growth limiting nutrient	DNP (M)	$\text{Na}_2\text{S}_2\text{O}_3$ ( $\mu\text{mole}$ )	$\text{Na}_2\text{S}$ ( $\mu\text{mole}$ )	Residual polythionates $\text{S}_4\text{O}_6^{2-}$ ( $\mu\text{mole}$ )	$\text{S}_2\text{O}_3^{2-}$ ( $\mu\text{mole}$ )	$\text{S}_3\text{O}_6^{2-}$ ( $\mu\text{mole}$ )	Gas uptake ( $\mu\text{mole}$ )	$^{14}\text{CO}_2$ fixation (cpm) *	Gas uptake per $\mu\text{mole}$ $\text{Na}_2\text{S}_2\text{O}_3$ or $\text{Na}_2\text{S}$ ( $\mu\text{mole}$ ) *	$^{14}\text{CO}_2$ fixation per $\mu\text{mole}$ $\text{Na}_2\text{S}_2\text{O}_3$ or $\text{Na}_2\text{S}$ ( $\mu\text{mole}$ ) *	Incubation time (mins)	Organism conc. (mg protein)
Thiosulphate	0	0	-	-	-	-	1.99	24,229	-	-	270	3.37
	$10^{-5}$	0	-	-	-	-	2.52	18,978	-	-		
	$10^{-4}$	0	-	-	-	-	3.08	13,755	-	-		
	$10^{-3}$	0	-	-	-	-	4.96	4,157	-	-		
	0	6	-	0	0.18	0	8.54	323,355	1.42	53,892	330	1.95
	$10^{-5}$	6	-	0	0	0	7.48	227,621	1.25	37,937		
	$10^{-4}$	6	-	0	0	0	8.22	141,885	1.37	23,648		
	$10^{-3}$	6	-	0.92	0	0	8.32	24,478	1.39	4,080		
	0	-	0	-	-	-	8.08	37,401	-	-	330	1.95
	$10^{-5}$	-	0	-	-	-	9.31	37,008	-	-		
Culphide	$10^{-4}$	-	0	-	-	-	9.64	31,754	-	-		
	$10^{-3}$	-	0	-	-	-	12.66	8,280	-	-		
	0	-	4	-	-	-	13.51	99,570	3.38	24,893		
	$10^{-5}$	-	4	-	-	-	16.27	99,829	4.07	24,957		
	$10^{-4}$	-	4	-	-	-	18.54	59,820	4.64	14,455	330	1.95
	$10^{-3}$	-	4	-	-	-	18.28	5,216	4.57	1,304		

\* 355,200 cpm = 1  $\mu\text{mol CO}_2$

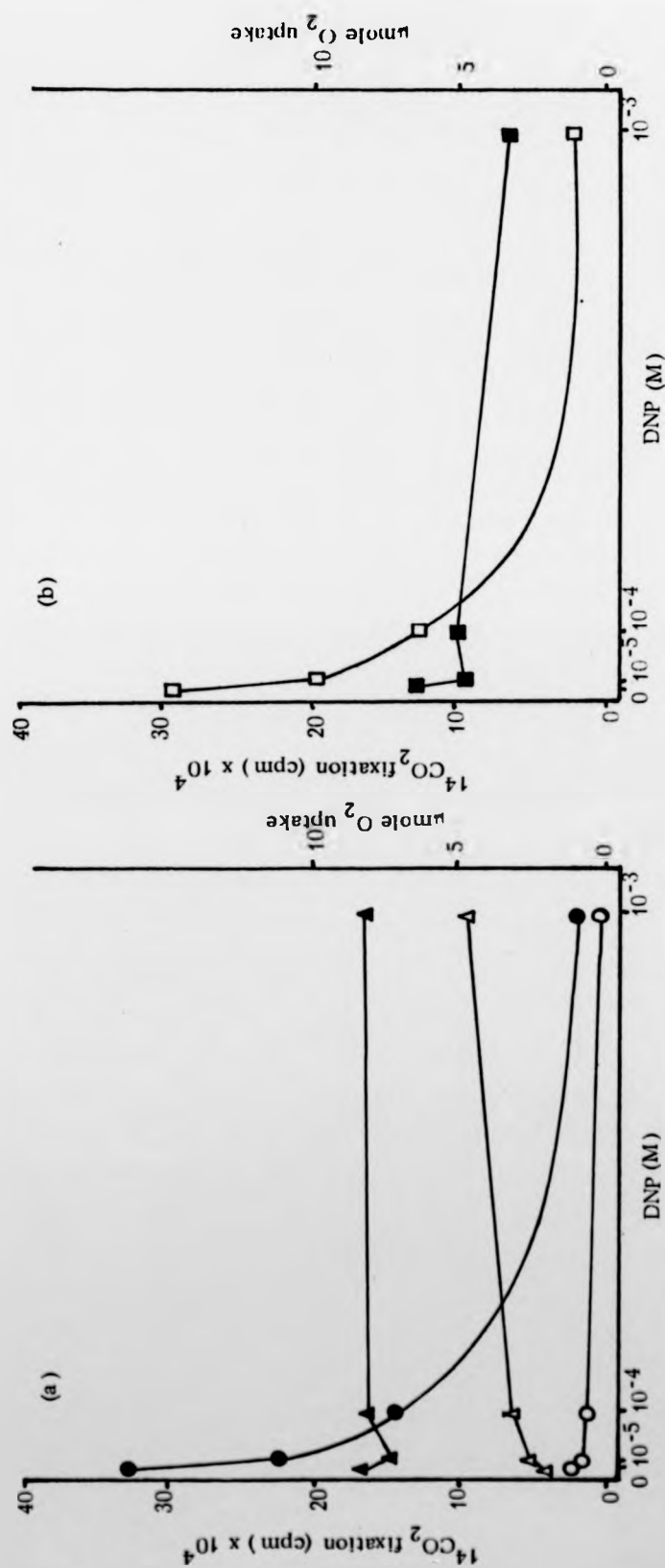


FIG. 32 Effect of 2,4-dinitrophenol on  $^{14}\text{CO}_2$  fixation and oxygen uptake coupled to aerobic thiosulphate oxidation by thin cell suspensions. (a)  $^{14}\text{CO}_2$  fixation without  $\text{Na}_2\text{S}_2\text{O}_3$  (O), with 6  $\mu\text{mole Na}_2\text{S}_2\text{O}_3$  (●); oxygen uptake without  $\text{Na}_2\text{S}_2\text{O}_3$  (Δ), with 6  $\mu\text{mole Na}_2\text{S}_2\text{O}_3$  (▲). (b)  $^{14}\text{CO}_2$  fixation (□) and oxygen uptake (■) corrected for reaction, absence of  $\text{Na}_2\text{S}_2\text{O}_3$ . Cell suspension = 3.37 mg protein.

TABLE 26 The effect of  $10^{-4}$  M 2,4-dinitrophenol on  $^{14}\text{CO}_2$  fixation and oxygen uptake coupled to aerobic thiosulphate and sulphide oxidation by thin cell suspensions

Growth limiting nutrient	DNP (M)	$\text{Na}_2\text{S}_2\text{O}_3$ ( $\mu\text{mole}$ )	$\text{Na}_2\text{S}$ ( $\mu\text{mole}$ )	End products $\text{SO}_4^{2-}$ $\text{S}_2\text{O}_3^{2-}$ $\text{S}_2\text{O}_4^{2-}$ $\text{S}_2\text{O}_6^{2-}$ $\text{S}_3\text{O}_6^{2-}$	Gas uptake ( $\mu\text{mole}$ )	$^{14}\text{CO}_2$ fixation (cpm) *	Corrected gas uptake ( $\mu\text{mole}$ )	Corrected $^{14}\text{CO}_2$ fixation (cpm) *	Organism concentration (mg protein)
Thiosulphate 210 mins	$10^{-4}$	6	-	15.32 0.64 0 0	16.56	124,148	3.1	102,761	5.08
	$10^{-4}$	0	-	- - - -	13.46	21,387	-	-	
	0	6	-	19.53 0.64 0 0	15.39	192,193	6.27	161,560	
	0	0	-	- - - -	9.12	30,633	-	-	
Sulphide 210 mins	$10^{-4}$	-	4	7.66 - - -	18.61	51,564	4.98	28,446	5.08
	$10^{-4}$	-	0	- - - -	13.63	23,118	-	-	
	0	-	4	8.04 - - -	15.14	106,615	5.74	81,213	
	0	-	0	- - - -	9.40	25,402	-	-	

\* 355,200 cpm  $\equiv$  1  $\mu\text{mol CO}_2$

TABLE 27 The effect of  $10^{-4}$  M 2,4-dinitrophenol on  $^{14}\text{CO}_2$  fixation and oxygen uptake coupled to aerobic thiosulphate and sulphide oxidation by starved and unstarved thin cell suspensions

Growth limiting nutrient	Starved or unstarved cells	$\text{Na}_2\text{S}_2\text{O}_3$ ( $\mu\text{mole}$ )	$\text{Na}_2\text{S}$ ( $\mu\text{mole}$ )	Gas uptake ( $\mu\text{mole}$ )	$^{14}\text{CO}_2$ fixation (cpm) *	Corrected gas uptake ( $\mu\text{mole}$ )	Corrected $^{14}\text{CO}_2$ fixation (cpm) *	Incubation time (mins)	Organism concentration (mg protein)
Thiosulphate	unstarved	0	-	13.46	21,387	-	-	210	5.08
		6	-	16.56	124,148	3.1	102,761	210	5.08
	starved	0	-	3.33	5,247	-	-	270	3.35
		6	-	8.65	247,958	5.32	242,711	270	3.35
Sulphide	unstarved	-	0	13.63	23,118	-	-	210	5.08
		-	4	18.61	51,564	4.98	28,446	210	5.08
	starved	-	0	2.45	5,034	-	-	270	3.35
		-	4	9.26	133,957	6.81	128,923	270	3.35

\* 355,200 cpm  $\equiv$  1  $\mu\text{mol CO}_2$

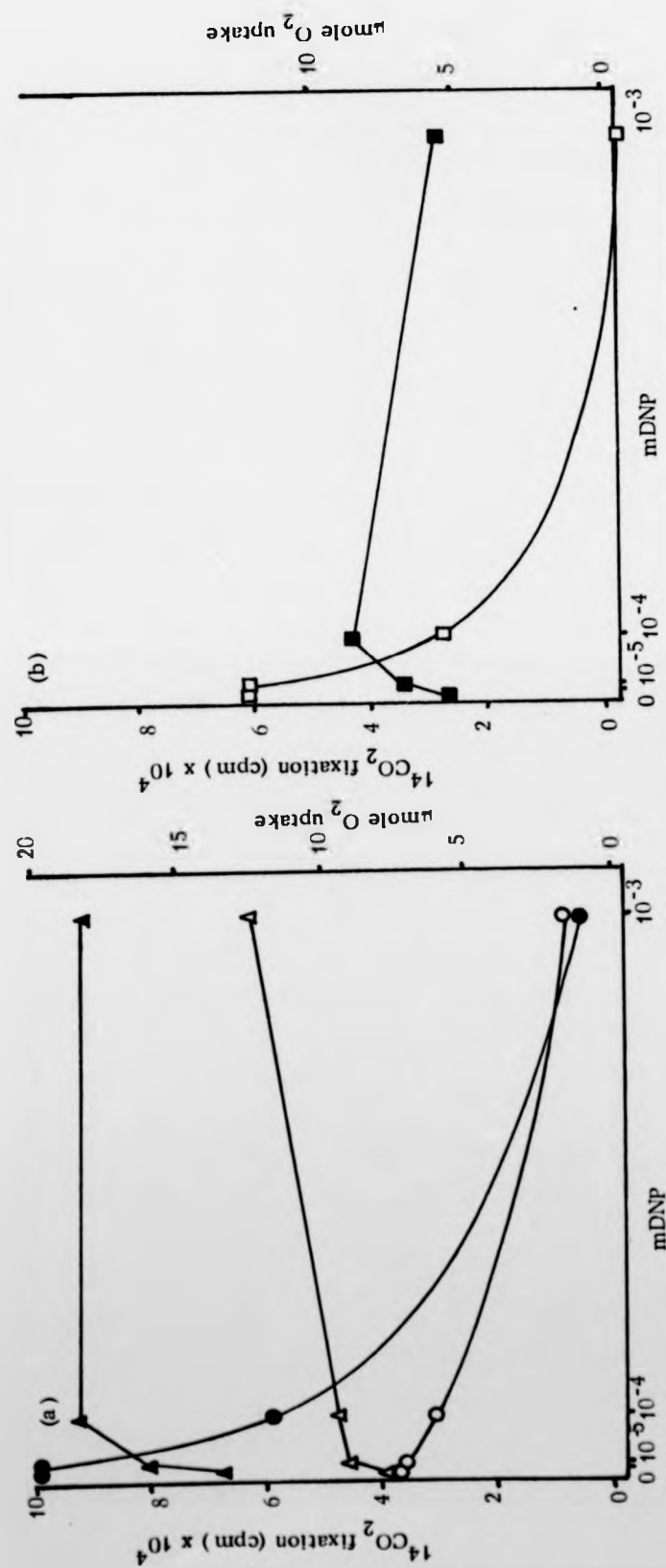


FIG. 33 Effect of 2,4-dinitrophenol on  $^{14}\text{CO}_2$  fixation and oxygen uptake coupled to aerobic sulphide oxidation by thin cell suspensions.

(a)  $^{14}\text{CO}_2$  fixation without  $\text{Na}_2\text{S}$  (○), with 4  $\mu\text{mole Na}_2\text{S}$  (●); oxygen uptake without  $\text{Na}_2\text{S}$  (△), with 4  $\mu\text{mole Na}_2\text{S}$  (▲).

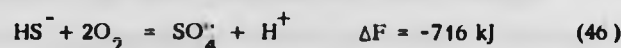
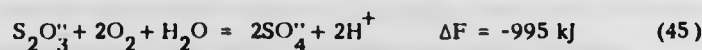
(b)  $^{14}\text{CO}_2$  fixation (□) and oxygen uptake (■) corrected for reaction in absence of  $\text{Na}_2\text{S}$ . Cell suspension = 1.95 mg protein.

#### 5.4 Discussion

Carbon dioxide fixation by *T. denitrificans* takes place chiefly by the Calvin cycle (Trudinger, 1955, 1956; Peeters and Aleem, 1970a; Taylor, Hoare and Hoare, 1971) and requires a supply of NADH and ATP.

Aerobically, carbon dioxide fixation, with thiosulphate as the energy source was inhibited at  $10^{-5}$ ,  $10^{-4}$  and  $10^{-3}$  M DNP by 29.6%, 56.1% and 92.4% respectively, and with sulphide as substrate by 39.9% and 94.8% with  $10^{-4}$  and  $10^{-3}$  M DNP. These values are not corrected for the reaction in the absence of the energy source. Taking this into account, these values become 30.3%, 57.2% and 93.2% with thiosulphate, and 54.9%, 100% with sulphide respectively. As carbon dioxide fixation is inhibited by low concentrations of DNP, the amount of carbon dioxide fixed could indicate the quantity of high energy phosphate available to the organism.

Less carbon dioxide was fixed per molecule of substrate oxidized when sulphide was the substrate instead of thiosulphate. The quantity of carbon dioxide fixed during the oxidation of one mole sulphide was 59.3% of that fixed when one mole thiosulphate was oxidized aerobically (or 61.6% when absence of thiosulphate was taken into account). This was calculated on comparison of experiments with equimolar quantities of sulphide and thiosulphate (6  $\mu$ mole). The oxidation of 1 mole sulphide and 1 mole thiosulphate both require 2 mole oxygen, but give different free energy yields.



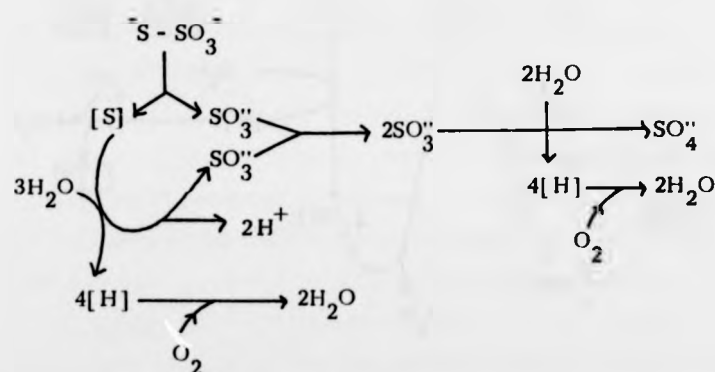
If all oxygen consumption were limited to oxidative phosphorylation with a P/O ratio of 1.0, equal ATP synthesis might be expected to occur with sulphide or thiosulphate. Thermodynamically, the oxidation of sulphide should, however, produce only 72% of the energy

available from thiosulphate oxidation. Hence the <sup>14</sup>CO<sub>2</sub> difference in the amount of fixed would be expected to be in this region. Low concentrations of dinitrophenol inhibited carbon dioxide fixation, but not the oxidation of thiosulphate or sulphide. This suggests that oxidative phosphorylation is involved in coupling between carbon dioxide fixation and substrate oxidation (Kelly and Syrett, 1963). The difference in the amount of carbon dioxide fixed by sulphide and thiosulphate (assuming this indicates the quantity of high energy phosphate made available during oxidation) suggests that the oxidation of a molecule of thiosulphate is linked to more phosphorylating steps than that of one of sulphide. The additional process of substrate level phosphorylation (Peck, 1962) demonstrated in T. denitrificans (Bowen et al., 1966; Sargeant et al., 1966; Aminuddin and Nicholas, 1974a) is not uncoupled by 2, 4-dinitrophenol. Substrate-level phosphorylation is not more important in supplying energy for carbon dioxide fixation during the oxidation of thiosulphate than during sulphide oxidation because there was no significant difference in sensitivity to dinitrophenol by both of them. Thus the additional energy available from thiosulphate oxidation could come from oxidative phosphorylation accompanying oxidation of sulphite derived from the sulphonate residue of thiosulphate.

The sites of phosphorylation in T. denitrificans have not been looked into, so the sites available for energy conservation can only be speculative. Sites have been established in other Thiobacilli (Kelly and Syrett, 1966a); Aleem (1966) postulated the existence of three phosphorylation sites in T. novellus associated with autotrophic growth; Drozd (1974) postulated one site of energy conservation in T. neapolitanus between cytochrome c and oxygen associated with thiosulphate with a maximum P/O ratio of 0.8-0.9. The ATP production from the inhibition studies on T. denitrificans was not measured, but the Y<sub>g</sub> calculations based on the chemostat



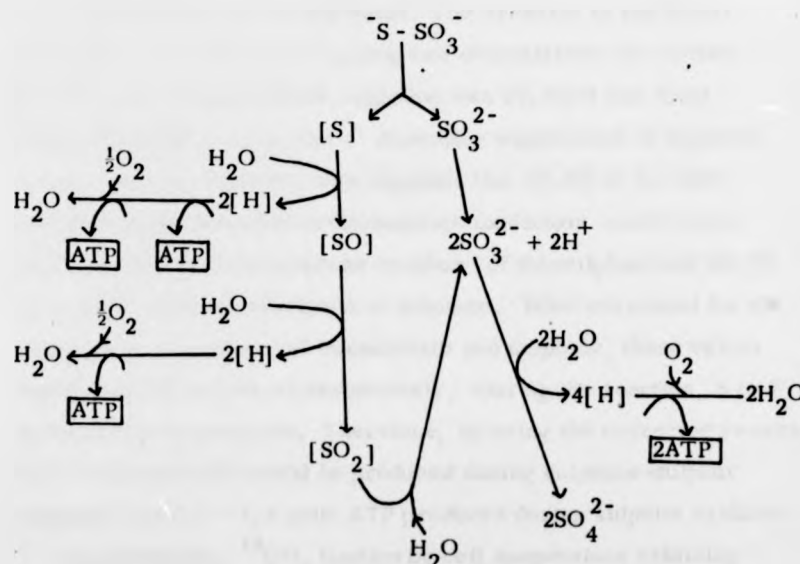
grown organisms enabled an estimation of ATP per mole of thiosulphate oxidized to be made, which was between 6 and 7 aerobically (on the assumption that 2 ATP was required per  $\text{NAD}^+$  reduced). If there was a P/O ratio of 1, a maximum of 4 ATP would be produced with 8 electrons released by the complete oxidation of 1 mole thiosulphate to sulphate, and passed via the electron transport chain and oxygen reduction coupled to oxidative phosphorylation:



Alternatively, after thiosulphate is split to sulphane-sulphur and sulphite, further oxidation to sulphate led to a possible P/O ratio of 1 and 2 respectively. 4 electrons produced via oxidations of sulphite and 8 electrons produced from sulphide giving a total value of 6 mole ATP.

A third possibility is for a P/O ratio of 1.5 for the sulphane-sulphur oxidation because of the decreasing redox for sequential oxidation to sulphite, leading to a total of 5 ATP:





So far, only oxidative phosphorylation has been considered. If substrate level phosphorylation is added into these three possible systems, the overall ATP produced would increase, as suggested by Vishniac and Santer (1957), Vishniac and Trudinger (1962) in thiolbacilli. Substrate level phosphorylation goes through a sequence of reactions (equations 20-24) from which energy is conserved by adenylate kinase producing 1 ATP from 2 ADP, so making the total ATP in the three possibilities mentioned 5, 7 or 6 respectively. During this sequence, 4 electrons are released during the production of APS from two sulphite ions which could undergo oxidative phosphorylation to produce 2 ATP. Hence from one sulphite molecule  $1\frac{1}{2}$  ATP could be produced, one generated by oxidative phosphorylation and  $\frac{1}{2}$  by substrate-level phosphorylation.

Another possibility is that substrate-level phosphorylation could be an alternative to oxidative phosphorylation accompanying sulphite oxidation, making a total ATP of 3, 5 or 4 respectively.

Based on the inhibition studies and the ATP production in the chemostat cultures, the ratio and distribution of the two types of phosphorylation could be predicted. The oxidation of equimolar quantities of sulphide and thiosulphate demonstrate that carbon dioxide fixed during sulphide oxidation was 59.3% of that fixed during thiosulphate oxidation. Assuming equivalence of sulphide and the sulphane-sulphur, this suggests that 59.3% of the total phosphorylation coupled to thiosulphate oxidations could occur during oxidation of the sulphane-sulphur of thiosulphate and 40.7% during the oxidation of sulphite to sulphate. When corrected for the reaction in the absence of thiosulphate and sulphide, these values become 61.6% and 38.4% respectively. During the reaction, 6 to 7 mole should be produced. Therefore, by using the corrected results, 3.7 - 4.3 mole ATP could be produced during sulphane-sulphur oxidation and 2.3 - 2.7 mole ATP produced during sulphite oxidation.

Anaerobically,  $^{14}\text{CO}_2$  fixation by cell suspensions oxidizing thiosulphate or sulphide was inhibited 95% by  $10^{-3}$  M DNP with thiosulphate as the energy source, and 80.3% with sulphide. Once corrected, these become 97.8% and 98.6% respectively. At lower DNP concentrations, carbon dioxide fixation increased by 13.2% and 30.7% at  $10^{-5}$  M and  $10^{-4}$  M with thiosulphate, and 28.5% and 23.6% respectively with sulphide. The corrected values are 14.8% and 32.2% with thiosulphate and 25% and 19.4% with sulphide respectively. This increase might be a result of the increased availability of orthophosphate due to DNP-stimulated phosphatases, or perhaps from the stimulation of endogenous metabolism by DNP (Peck, 1968). The quantity of carbon dioxide fixed during oxidation of one mole sulphide was 81.2% (corrected to 80.6%) of that fixed when one mole thiosulphate was oxidized anaerobically. This suggests that 80.6% of the total phosphorylation coupled to thiosulphate could occur during oxidation of sulphane-sulphur to sulphate and 19.4% during the oxidation of sulphite to sulphate. This figure is closer to the thermodynamic figure of 72% previously mentioned. The estimation of ATP

production based on the Yg data suggested that 4 to 5 mole ATP are produced anaerobically. Therefore, 3.22 - 4.03 mole ATP are produced during sulphane-sulphur oxidation, and 0.78 - 0.97 mole ATP are produced by the oxidation of sulphite. The possibility of calculating the distribution of ATP has been complicated by the incomplete reduction of nitrate to nitrite and possibly nitrous oxide, and the incomplete oxidation of thiosulphate. Cheah (1973) found nitrite inhibited both respiration and oxidative phosphorylation in mitochondrial respiration, and so the accumulation of nitrite may also lead to an unreliable interpretation of the results.

In comparison between anaerobic and aerobic results, more carbon dioxide was fixed by the oxidation of sulphide as a percentage of thiosulphate oxidation, anaerobically than aerobically. This suggests that the conservation of energy between sulphide and sulphite, anaerobically, was greater than that aerobically, in the absence of the sulphur-oxidizing enzyme, and that, anaerobically, less energy is conserved between sulphite and sulphate than aerobically.

PART 6: DISCUSSION AND GENERAL REMARKS

#### PART 6: DISCUSSION AND GENERAL REMARKS

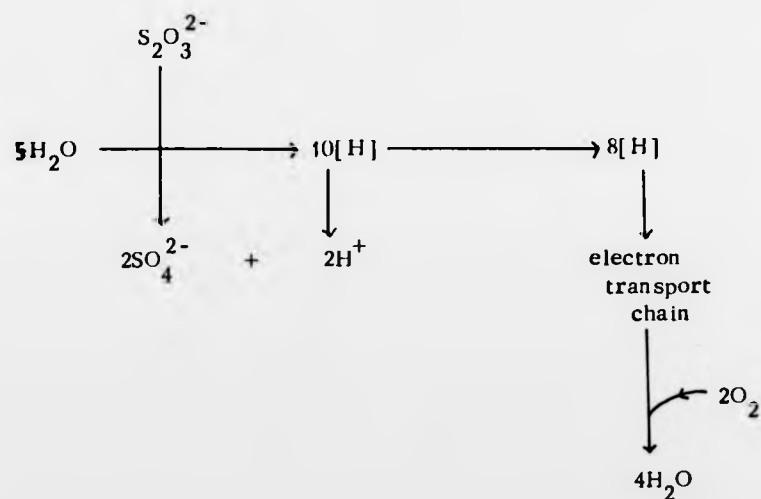
Thiobacillus denitrificans is a versatile chemolithoautotrophic bacterium. In this study it has been found capable of oxidizing thio-sulphate and tetrathionate as its energy source, with carbon dioxide as its carbon source, under chemostat conditions. It was also capable of using sulphur and sulphide in the cell suspension experiments. Under anaerobic conditions in the chemostat, it used nitrate, nitrite or nitrous oxide as an alternative electron acceptor to oxygen.

The energy released from the oxidation of the sulphur compounds was converted to biologically utilizable energy in the form of ATP by substrate level phosphorylation (Bowen, Happold and Taylor, 1966; Sargeant *et al.*, 1966; Aminuddin and Nicholas, 1974a) and by proton translocation along an electron transport system in oxidative phosphorylation (Aminuddin and Nicholas, 1974b; Tuovinen, Nicholas and Aleem, 1977). The total energy source consumed contributes partly to energy for growth and partly for maintenance. Some ATP may be lost by hydrolysis and thus not coupled to growth or maintenance functions: ATP lost in this way would be indistinguishable from the maintenance energy requirement, so raising this value. If the maintenance energy were zero, the maximum growth yield would be obtained from the energy source. In Section 3 the theoretical values for  $Y_g$  were derived, enabling the maximum amount of energy conserved to be estimated.

The energy transfer along the electron transport system anaerobically and aerobically involves the movement of  $8[H]$  from the oxidation of one mole thiosulphate (Scheme 3). The reduction of  $NAD^+$  via the electron transport pathway occurs via an 'uphill' reaction in all chemolithotrophs, except the hydrogen bacteria, at the expense of energy consumed during the 'downhill' transfer of electrons from cytochrome c to oxygen or nitrate (Kelly, 1978). So, in all the ATP calculations the energy required for  $NAD^+$  reduction was taken into account.

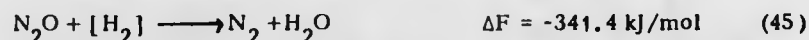
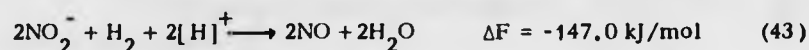
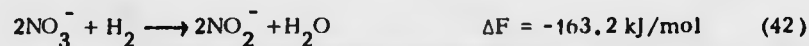
Chemolithotrophs such as *T. denitrificans* appear to have evolved a respiratory metabolism based on an oxidizable substrate to produce energy. The production of this free energy is as a result of the oxidation being highly exergonic, and that the amount of energy being released is enough to support ATP production. Hydrogen equivalents produced from the oxidation reduce  $\text{NAD}^+$  so that metabolic reduction reactions such as carbon dioxide fixation, and amination of 2-oxo-glutarate to glutamic acid can be supported (Kelly, 1978). The overall efficiency of conversion of energy (as available free energy) from oxidation reactions to metabolic energy as measured by the production of new bacterial growth was 30.06% anaerobically and 30.22% aerobically in *T. denitrificans*, showing that the production of energy is comparable with the efficiency of mitochondrial respiration and photosynthetic energy (Lehninger, 1975). Energy conservation by both types of phosphorylation has been demonstrated with the use of 2, 4-dinitrophenol, and indicates a greater proportion of phosphorylation during oxidation of sulphide anaerobically than aerobically, as compared with thio-sulphate.

In the chemostat studies the  $Y_g$  of 14.43 g dry wt. (g mole thio-sulphate) $^{-1}$  was greater than that anaerobically (11.35) indicating ATP production of 6 to 7 mole rather than 4 to 5 mole. The anaerobic  $Y_{\text{ATP}}$  was thus 2.27-2.84 g dry wt. (mole ATP) $^{-1}$ , and aerobically 2.06-2.41. Therefore 0.352-0.440 mole of ATP was required anaerobically during the synthesis of 1 g dry wt. of cells, and 0.415-0.485 mole ATP is required aerobically. It was evident that during thiosulphate oxidation the  $Y_{\text{ATP}}$  is not influenced by the nature of the respiratory oxidant. The oxidation of tetrathionate resulted in a  $Y_g$  nearly twice as great as with thiosulphate anaerobically, in agreement with the relative free energy available. 8 to 9 mole ATP made available per tetrathionate oxidized resulted in a  $Y_{\text{ATP}}$  of 2.39 to 2.69 g dry wt. (mole ATP) $^{-1}$ , and hence 0.372 to 0.418 mole ATP required during the synthesis of 1 g of dry wt. cells. So, not only was the  $Y_{\text{ATP}}$  not influenced by the nature of the respiratory oxidant, but also by the energy source, whether thiosulphate or tetrathionate.

SCHEME 3Oxidation of one mole thiosulphate to produce reducing equivalents

The difference in growth yields of aerobic and anaerobic thio-sulphate cultures may indicate a greater efficiency of energy conservation during the oxidation of sulphide to sulphate either by substrate level phosphorylation or oxidative phosphorylation aerobically. The electrons released from the oxidation of the sulphane-sulphur to sulphate passed along the electron transport system could involve the possible production of four ATP, two between cytochrome c and oxygen (or nitrate). The oxidation of the sulphite moiety to sulphate could involve the production of a further two ATP. The extra ATP produced aerobically could be due to the two coupling sites in sulphane-sulphur oxidation occurring and only one such site anaerobically (Scheme 4). This could depend on where nitrate and nitrite takes the electrons from. According to Aminuddin and Nicholas (1973), sulphide oxidation to sulphite, anaerobically, requires nitrite as oxidant, and from sulphite to sulphate nitrate or nitrite is required.

Taking thermodynamic considerations, each of the four reactions from nitrate to nitrogen could be coupled to phosphorylation (Thauer, Jungermann and Decker, 1977).

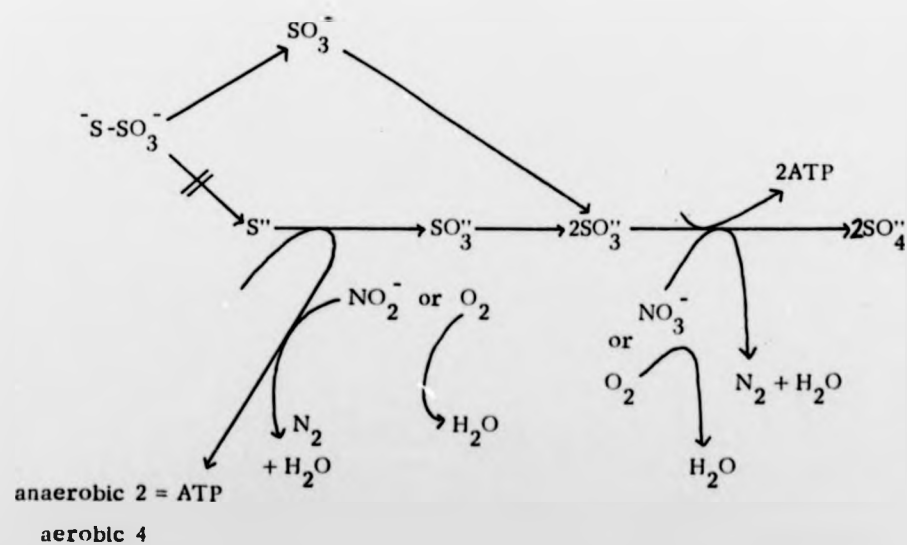
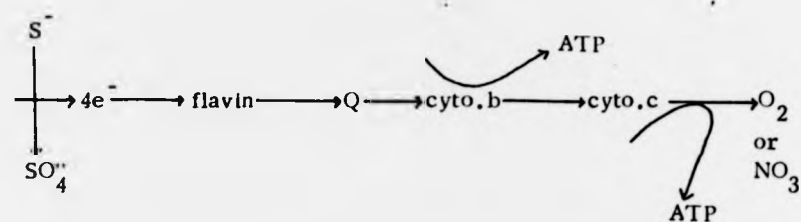


The molar growth yields of a variety of bacteria grown anaerobically with nitrate as a terminal electron acceptor indicates that phosphorylation coupled to nitrate reduction is a common phenomenon in bacteria containing particle bound nitrate reductase, cytochromes and a quinone participate in the electron transport to nitrate, e.g. *Micrococcus denitrificans* (John and Whatley, 1970), *T. denitrificans* (Sawhney and Nicholas, 1977). The free energy change of the three remaining reactions are large enough for the synthesis of ATP at each stage. The sites of phosphorylation on the remaining pathway vary with different



## SCHEME 4

Possible sites of ATP production from thiosulphate oxidation



organisms. For example, Micrococcus denitrificans (Naik and Nicholas, 1966) observed that nitrate and nitrite were involved in phosphorylation but not nitric oxide or nitrous oxide, whereas Pseudomonas denitrificans (Koike and Halton, 1975) found phosphorylation coupled to nitrate, nitric oxide and nitrous oxide reduction while Oknishi and Mori (1960) found it coupled to nitrite. Pseudomonas perfectiformis (Cox and Payne, 1973) the reduction of nitrate and nitrous oxide were concluded to be energy yielding systems.

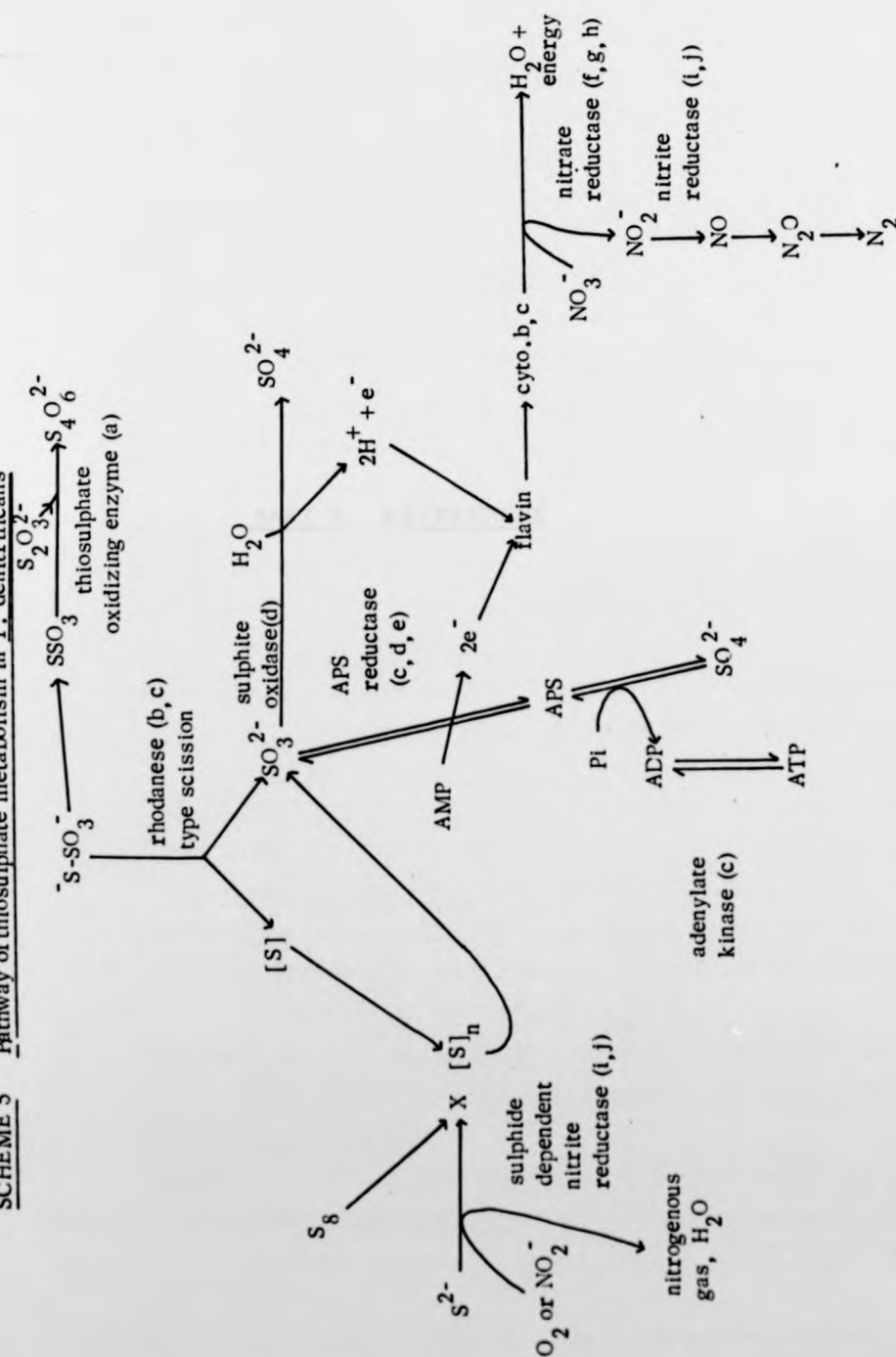
The presence of nitrate reductase activity during nitrous oxide reduction in T. denitrificans but absence during nitrite reduction indicates that this enzyme is under strict control by nitrate, and that the reduction of nitrate to nitrite may be the limiting step in denitrification. Nitrite in high quantities can be inhibitory (Timmer-ten Hoor, 1976), hence its rapid reduction would be beneficial to the organism. The repression of nitrate reduction in nitrite-grown cultures indicates that this may be a mechanism by which all the nitrite was reduced before any more could be produced from nitrate. The ability of T. denitrificans to reduce nitrous oxide demonstrates the end product in nitrate reduction is nitrogen and not nitrous oxide as in some other denitrifying bacteria such as Pseudomonas fluorescens and Pseudomonas chlororaphis (Greenberg and Becker, 1977).

The metabolism of sulphur compounds has been studied in T. denitrificans to enable a possible scheme of thiosulphate oxidation to be produced (Scheme 5). The use of the chemostat has produced results, not only in agreement to this but also enabled the estimation of energy conservation involved. The very low sulphur-oxidizing enzyme activity and production of ATP during the oxidation of sulphide to sulphate, as well as the high specific rates of thiosulphate consumption, suggests that another energy producing reaction occurs at this point that has not yet been found. Also there is no obvious role for the sulphur-oxygenase activity in anaerobic bacteria, unless this enzyme has another as yet undetected function.

Reference key to Scheme 5

- a = Justin and Kelly (1978)
- b = Bowen, Butler and Happold (1965)
- c = Sargeant et al. (1966)
- d = Aminuddin and Nicholas (1974a)
- e = Bowen, Happold and Taylor (1966)
- f = Adams, Warnes and Nicholas (1971b)
- g = Sawhney and Nicholas (1977)
- h = Sawhney and Nicholas (1978b)
- i = Aminuddin and Nicholas (1973)
- j = Sawhney and Nicholas (1978a)

SCHEME 5 Pathway of thiosulphate metabolism in *T. denitrificans*



For references, see opposite page.

PART 7: REFERENCES

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APPENDIX: REPRINTS OF PUBLICATIONS

G14 Metabolic Changes Accompanying Transition of Chemostat Cultures of *Thiobacillus denitrificans* from Aerobic to Anaerobic Growth. By PAULINE JUSTIN and D.P. KELLY (Department of Environmental Sciences, University of Warwick, Coventry, CV4 7AL).

*T. denitrificans* can be grown in continuous culture (1) both aerobically (thiosulphate-limited) and anaerobically (nitrate or nitrite-limited). An aerobic shake-flask culture was used to initiate a chemostat culture in a medium containing 20 mM  $\text{Na}_2\text{S}_2\text{O}_4$  + 20 mM  $\text{KNO}_3$ . Dissolved oxygen was initially poised at 88% of air saturation (about  $200 \mu\text{M O}_2$ ) by passage of air containing 5% (v/v)  $\text{CO}_2$  at about  $250 \text{ ml min}^{-1}$ . A steady state was established at  $D = 0.08 \text{ h}^{-1}$  and biomass, thiosulphate, nitrate and nitrite measured. Subsequently steady states were established at ten progressively lower dissolved oxygen levels and finally under complete anaerobiosis by progressive replacement of air by 5% carbon dioxide in nitrogen. At all dissolved oxygen values above 0% no thiosulphate was detectable, no significant loss of nitrate occurred; and nitrite was about 0.02 mM above 9% air saturation but increased 6-8 times at 8.7 and 5.2%. Anaerobically, essentially complete nitrate consumption ensued and transitory accumulation of sulphur and nitrite occurred during transition to steady state. At steady state, sulphur and nitrite were absent and about 2.6 mM thiosulphate remained (Cf ref 1). Aerobic metabolism was reestablished without lag when the anaerobic culture was aerated to 80% air saturation.

Growth yields increased with decreasing oxygen as follows: 7.75 g dry wt (g mole thiosulphate)<sup>-1</sup> at 88% air saturation, 7.38 (78%), 7.56 (68%), 10.06 (55%), 9.82 (44%), 10.54 (30%), 10.58 (22%), 11.56 (8.7%), 12.34 (5.2%), but fell to 8.07 anaerobically as expected from energetic considerations.

Nitrite reduction with thiosulphate by washed suspensions from different steady states was preceded by a lag determined by the steady state dissolved oxygen concentration and decreased progressively from 140 min (55%) to 30 min (8.7%) to no lag at 0%, but cultures at  $\geq 68\%$  did not attack nitrite in 300 min.

Nitrate and nitrite reductase activities in crude cell-free extracts (unit =  $\mu\text{mole min}^{-1} \text{ mg protein}^{-1}$ ) were 0.157 and 0.118 (88% air saturation), 0.128 and 0.125 (72%), 0.133 and 0 (60%), 0.172 and 0 (52%), 0.118 and 2.94 (37%), 0.156 and 6.17 (35%), 0.222 and 4.85 (22%), 0.396 and 8.59 (16%), 0.897 and 4.74 (8.7%), 1.885 and 26.90 (0%). For comparison, a nitrate-limited anaerobic steady state (in nitrate-free medium) contained 0.197 units nitrate reductase but 28 units nitrite reductase, while a nitrate-limited steady state with 10 mM  $\text{K}_2\text{S}_2\text{O}_8$  replacing thiosulphate contained 1.58 and 24.1 units nitrate and nitrite reductases respectively.

*T. denitrificans*, an obligate chemolithotroph, thus resembles other facultative anaerobes in exhibiting strong repression control by oxygen of nitrate reduction, and shows nitrate-dependent anaerobic nitrate reductase synthesis. Oxygen is a somewhat inhibitory substrate, depressing the growth yield at all but low concentrations.

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Growth of *Thiobacillus denitrificans* in Continuous-Flow Culture. By PAULINE JUSTIN and D.P. KELLY (Department of Environmental Sciences, University of Warwick, Coventry CV4 7AL).

Culture of aerobic sulphur bacteria in thiosulphate-limited chemostats enables estimation of true growth yields ( $Y_G$ ) and maintenance coefficients ( $m$ ) by standard procedures (1). Work with *Thiobacillus neapolitanus* (2) indicated  $Y_G$  (g dry wt/g mole thiosulphate<sup>-1</sup>) 13.9 and 7.7;  $m$  (mmole  $S_2O_3^{2-}$ /g dry wt<sup>-1</sup> h<sup>-1</sup>) 21.8 and 10.5. Lower  $Y_G$  values were indicated by later work (3,4) with *T. neapolitanus* (5.27, 6.5), *Thiobacillus A2* (ca 7) and *Thiomicrospira pelophila* (5.2). Similarly  $m$  was <1–2.6, although Kelly (3) obtained 28.2 for *T. neapolitanus*.

Anaerobic batch cultures of *T. denitrificans* NCIB 9548 on thiosulphate (respiratory and carbon dioxide reductant) and nitrate (respiratory oxidant) accumulated nitrite and sulphur, making measurements of energy-coupling efficiency imprecise. A chemostat culture (750 ml) was maintained in several steady states ( $D = 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08$  h<sup>-1</sup>) for 1801 h at 30°C, pH 6.9 ± 0.1, with stirring (750 rpm) and gassing with 5% (v/v) CO<sub>2</sub> in N<sub>2</sub> (10 ml min<sup>-1</sup>). The growth-limiting nutrient was KNO<sub>3</sub> (20mM), the amount of Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> oxidised depended on KNO<sub>3</sub> available and the dilution rate. Steady-state Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>(s) was 1–3.5 mM with negligible nitrate, nitrite, polythionates or sulphur. Steady-state biomass (measured as absorbance at 460 nm and as protein) increased with increased  $D$ . Plotting  $Y^{-1}$  (g g<sup>-1</sup> mole Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> oxidised<sup>-1</sup>) against  $D^{-1}$  gave parallel linear plots for dry wt and protein, indicating  $Y_G$  11.63 and 8.51 respectively; and  $m$  1.4. The specific rate of thiosulphate oxidation (mmoles/g dry wt<sup>-1</sup>) increased linearly from 2.85 at a  $D$  of 0.02 to 8.24 at a  $D$  of 0.08, extrapolating to 1.17 at zero growth rate ( $D = 0$ ). The high  $Y_G$  and low  $m$  values for anaerobic *T. denitrificans* may indicate better energy-coupling than in aerobic thiobacilli. This is being tested with thiosulphate-limited aerobic and anaerobic chemostats.

1. Pirt, S.J. (1965) *Proceedings of the Royal Society* B163, 224.
2. Hemphing, W.P. and Vishniac, W. (1967) *Journal of Bacteriology* 93, 874.
3. Kelly, D.P. Unpublished observations.
4. Kuenen, J.G. Personal communication.

## Growth Kinetics of *Thiobacillus denitrificans* in Anaerobic and Aerobic Chemostat Culture

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*Thiobacillus denitrificans* was cultured chemolithotrophically under aerobic and anaerobic conditions in a chemostat with thiosulphate, nitrate or nitrite as limiting nutrient. Estimations of growth yields and maintenance coefficients showed that *T. denitrificans* grew more efficiently than other thiobacilli both aerobically and anaerobically. Relative growth yield data enabled the probable amounts of ATP generated during thiosulphate-limited aerobic growth and nitrate-limited anaerobic growth on thiosulphate to be calculated as, respectively, 6 to 7 and 4 to 5 mol ATP formed per mol thiosulphate oxidized. The energy available from tetrathionate oxidation was almost twice that from thiosulphate.

### INTRODUCTION

*Thiobacillus denitrificans* is an obligate chemolithotroph which uses the oxidation of reduced inorganic sulphur compounds for the respiratory reduction of oxygen or, under anaerobic conditions, of nitrate to dinitrogen gas. It is capable of wholly autotrophic growth, effecting reductive fixation of carbon dioxide by the Calvin cycle under both aerobic and anaerobic conditions. It is the only well established example of a facultatively anaerobic thiobacillus and has been subject to considerable physiological and biochemical study largely concerned with batch cultures and the mechanisms of sulphur compound oxidation and nitrate reduction (Beijerinck, 1904; Lieske, 1912; Baalsrud & Baalsrud, 1952, 1954; Aubert, Millet & Milhaud, 1959; Woolley, Jones & Happold, 1962; Bowen, Butler & Happold, 1965; Bowen, Happold & Taylor, 1966; Sargeant *et al.*, 1966; Adams, Warnes & Nicholas, 1971; Taylor, Hoare & Hoare, 1971; Aminuddin & Nicholas, 1973, 1974*a, b*; Schedel, Legall & Baldensperger, 1975; Sawhney & Nicholas, 1977). Earlier claims that it lost its capacity for anaerobic growth following culture aerobically (Vishniac & Santer, 1957; Woolley *et al.*, 1962) have never been substantiated and only recently has any attempt been made to study the bioenergetics of this organism by means of continuous chemostat culture (Justin & Kelly, 1976; Timmer-ten-Hoor, 1976).

This study was performed to seek information on the behaviour of continuous cultures of *T. denitrificans* subject to substrate limitation under aerobic and anaerobic conditions and to determine the ease with which adaptation between aerobiosis and anaerobiosis could occur. The results enable calculation of comparative growth yields and 'maintenance energy' requirements under a variety of physiological steady state conditions.

### METHODS

**Organism and culture conditions.** *Thiobacillus denitrificans* NCIB 9548 was maintained anaerobically in completely filled bottles or aerobically in flasks shaken at 30 °C. Anaerobic nitrate-limited cultures and aerobic thiosulphate-limited cultures were grown in a medium containing (g l<sup>-1</sup> in distilled water): Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>, 5H<sub>2</sub>O, 5; KNO<sub>3</sub>, 2; KH<sub>2</sub>PO<sub>4</sub>, 2; NH<sub>4</sub>Cl, 1; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.4; FeSO<sub>4</sub>·7H<sub>2</sub>O, 1 ml of 2% (w/v) solution in



1 M-HCl; trace metal solution (Tuovinen & Kelly, 1973), 1 ml; adjusted to pH 7.0. For anaerobic growth on nitrite-limited medium,  $\text{KNO}_3$  was omitted from the medium and a solution of  $\text{NaNO}_2$  was separately pumped into the chemostat apparatus to avoid chemical reaction in the bulk medium between nitrite and thiosulphate. Similarly to obtain cultures growing on tetrathionate, a thiosulphate-free basal medium feed at pH 9.2 was employed together with a separate supply of  $\text{K}_2\text{S}_2\text{O}_8$  solution at pH 3.5, the two mixing in the chemostat vessel and being automatically maintained at pH 7.0. For anaerobic thiosulphate-limitation,  $\text{KNO}_3$  was supplied at  $3 \text{ g l}^{-1}$ .

Two types of chemostat were used. Most of the studies were carried out in an LH modular type series 500 fermenter (LH Engineering, Slough, Buckinghamshire) with a culture volume of 750 ml, provided with agitation ( $750 \text{ rev. min}^{-1}$ ), temperature control ( $30^\circ \text{C}$ ), pH control, aeration and dissolved oxygen monitoring modules. Some aerobic cultures were grown in an LH CC 1500 fermenter with a working volume of 3 l and control of temperature, pH, stirring and aeration. Culture pH was maintained at pH 7.0 by automatic titration with 0.5 M-NaOH. Anaerobic cultures were continuously flushed with 5% (v/v)  $\text{CO}_2$  in  $\text{N}_2$  at  $15 \text{ ml min}^{-1}$  for a 750 ml culture and the medium reservoir vessels were held under  $\text{N}_2$ . The effluent gas from the culture was passed through a Drechsel bottle trap containing alkaline pyrogallol. Medium solutions were pumped into the vessel through silicone or black butyl rubber tubing (for aerobic and anaerobic cultures, respectively) by means of Watson Marlow MHRE7 flow inducers (Watson Marlow, Falmouth, Cornwall). Aerobic cultures were poised at selected concentrations of dissolved oxygen by controlling the flow rate into the culture of air containing 5% (v/v)  $\text{CO}_2$ . Possible wall growth in the culture vessels was minimized by coating them with dichlorosilane, applied as a 5% solution in chloroform.

**Collection of gases from chemostat.** The effluent gas from a thiosulphate-limited anaerobic culture was passed through a U-tube packed with molecular sieve beads and cooled in liquid nitrogen. The condensed gases were revaporized and analysed for NO and  $\text{N}_2\text{O}$  by gas chromatography.

**Analysis of steady state cultures.** Samples were removed from steady state cultures at regular intervals for chemical and microbiological analysis. Biomass was measured as  $A_{660}$  using appropriate dry weight-absorbance calibration curves. (Direct determination of dry weights of organisms in cultures at different dilution rates confirmed that this was a reliable method for monitoring biomass concentration, but showed that a different relationship existed for aerobic and anaerobic cultures.)  $A = 0.5$  was equivalent to  $130 \text{ mg dry wt l}^{-1}$  in an anaerobic culture but to  $175 \text{ mg dry wt l}^{-1}$  in an aerobic culture: this relationship was constant up to  $A = 1.0$ . For estimation of protein, organisms from 4 ml samples were centrifuged, washed with distilled water, recentrifuged and finally heated in a boiling water bath for 10 min after adding 2.5 ml 0.5 M-NaOH. Solubilized protein was then determined (Lowry *et al.*, 1951). Microbiological purity of steady state cultures was checked by plating  $10^{-8}$  dilutions of cultures on (i) agar medium of the same composition as the liquid culture; (ii) agar medium as in (i) supplemented with 0.1% (w/v) glucose and 0.65% (w/v) nutrient broth; or (iii) agar with glucose and nutrient broth only.

Supernatant liquids from culture samples were analysed for their content of thiosulphate, trithionate and tetrathionate (Kelly, Chambers & Trudinger, 1969). Nitrate (0.03 to 3 mg) was determined by titration with 1.67 mM- $\text{K}_2\text{Cr}_2\text{O}_7$  using 0.025 M-ferroin as indicator (Kolthoff & Belcher, 1957). Nitrite was determined by the Griess-Ilosvay method: samples containing up to  $0.3 \mu\text{mol NO}_2^-$  were mixed with 1 ml 0.3 M- $\text{CdSO}_4$  and 1 ml reagent [equal volumes of 0.7% (w/v) sulphanilic acid in 30% (v/v) acetic acid and of 0.1 g  $\alpha$ -naphthylamine boiled in 20 ml water then supplemented with 150 ml 30% acetic acid], and made up to 10 ml with water; absorbance at 530 nm was read after 25 min. To overcome interference by thiosulphate with the assays of both nitrate and nitrite, all samples were supplemented with  $\text{Na}_2\text{S}_2\text{O}_8$  to a total concentration of 12 mM and all calibration curves were prepared with standard nitrate and nitrite containing 12 mM-thiosulphate. Any sulphur in the centrifuged pellet samples was redissolved in acetone and estimated colorimetrically (Bartlett & Skoog, 1954).

**Elemental analyses.** Organisms were harvested by centrifuging, washed and dried at  $105^\circ \text{C}$  before analysis for carbon, hydrogen and nitrogen using a Perkin-Elmer elemental analyser.

## RESULTS

### Anaerobic nitrate-limited chemostat culture

Commencing with a late exponential phase batch culture continually flushed with  $\text{N}_2/\text{CO}_2$ , a continuous culture was established without difficulty using a medium containing approximately 20 mM-thiosulphate and 20 mM-nitrate. Nitrate was the limiting nutrient in this medium because the stoichiometry for anaerobic thiosulphate respiration by *T. denitrificans* is given by:

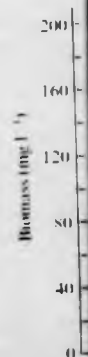


Fig. 1. Steady state culture on thiosulphate and protein.

Steady state Washout occurred at a dilution rate of  $0.24 \text{ h}^{-1}$  and the biomass concentration of the input medium was  $3 \text{ mm-thiosulphate}$ . Biomass and protein were  $7.03 \text{ g dry wt l}^{-1}$  and  $3.7 \text{ g protein l}^{-1}$  respectively. The maintenance of thiosulphate was  $1.17 \text{ mm-thiosulphate}$ .

The specific growth rate and the dilution rate were  $0.024 \text{ h}^{-1}$  and  $0.024 \text{ h}^{-1}$  respectively. The plot of  $\ln A_{660}$  against  $D$  was a straight line with a slope of  $1.17$  and an intercept of  $1.29$  on the  $D$ -axis.

A typical example of the effect of thiosulphate concentration on the growth rate is shown in Figure 1. The theoretical maximum biomass is, however,

1 M-HCl; trace metal solution (Tuovinen & Kelly, 1973), 1 ml; adjusted to pH 7.0. For anaerobic growth on nitrite-limited medium,  $\text{KNO}_3$  was omitted from the medium and a solution of  $\text{NaNO}_2$  was separately pumped into the chemostat apparatus to avoid chemical reaction in the bulk medium between nitrite and thiosulphate. Similarly to obtain cultures growing on tetrathionate, a thiosulphate-free basal medium feed at pH 9.2 was employed together with a separate supply of  $\text{K}_2\text{S}_2\text{O}_8$  solution at pH 3.5, the two mixing in the chemostat vessel and being automatically maintained at pH 7.0. For anaerobic thiosulphate-limitation,  $\text{KNO}_3$  was supplied at  $3 \text{ g l}^{-1}$ .

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Biomass ( $\text{mg l}^{-1}$ )

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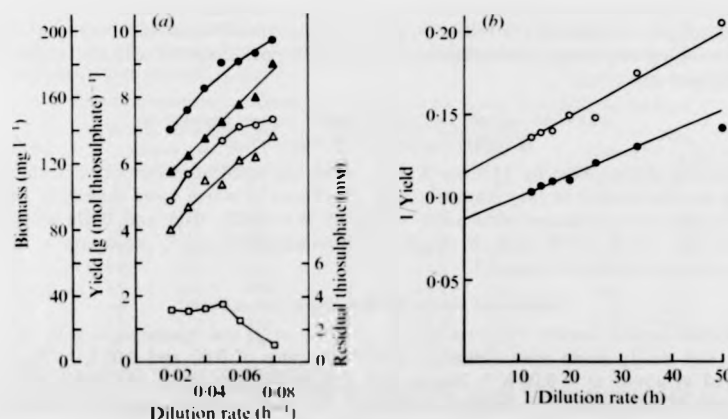


Fig. 1. Steady state biomass and yield of *Thiobacillus denitrificans* grown in anaerobic chemostat culture on thiosulphate (20 mM) with nitrate (23 mM) limitation. (a) Biomass (mg l<sup>-1</sup>) as dry wt (▲) and protein (△); yield [g (mol thiosulphate oxidized)<sup>-1</sup>] as dry wt (●) and protein (○); unoxidized thiosulphate (□). (b) Double reciprocal plots of dilution rates and yields: 1/[g dry wt (mol thiosulphate oxidized)<sup>-1</sup>] (●) and 1/[g protein (mol thiosulphate oxidized)<sup>-1</sup>] (○).

Steady states were maintained at seven dilution rates ( $D$ ) between 0.02 and 0.08 h<sup>-1</sup>. Washout occurred at  $D = 0.09$  h<sup>-1</sup>. Precise determination of input concentrations of thiosulphate and nitrate and steady state residual concentrations of these and of biomass enabled estimation of steady state yields in terms of g dry wt (or g protein) per mol thiosulphate (or nitrate) consumed. Residual nitrate was almost undetectable and was no more than 5 to 7% of the input concentration. Nitrite, sulphur and polythionates were not accumulated. About 3 mM-thiosulphate remained at low  $D$  values, decreasing to 1 mM at  $D = 0.08$  h<sup>-1</sup> (Table 1). Biomass and yield increased with increased dilution rate (Fig. 1a). The yield increased from 7.03 g dry wt per mol thiosulphate oxidized at  $D = 0.02$  h<sup>-1</sup> to 9.7 at  $D = 0.08$  h<sup>-1</sup>. The mean protein content (% w/w, of dry wt) did not vary with dilution rate and was  $75.8 \pm 3.7\%$  (S.E.M. of seven dilution rates). Plotting reciprocals of yields against reciprocals of  $D$  produced linear graphs (Fig. 1b) from which the true growth yield ( $Y_0$ ) and apparent maintenance coefficient ( $m$ ) could be calculated (Pirt, 1965).  $Y_0$  was 11.63 g dry wt (mol thiosulphate)<sup>-1</sup> or 8.51 g protein mol<sup>-1</sup>, and  $m$  was 1.4 mmol thiosulphate h<sup>-1</sup> (g dry wt)<sup>-1</sup>.

The specific rate of thiosulphate oxidation [ $q$ , in mmol h<sup>-1</sup> (g dry wt)<sup>-1</sup>] in the steady state chemostat cultures was calculated for each dilution rate from the thiosulphate consumption rate and the steady state biomass. The value of  $q$  increased from 2.85 at  $D = 0.02$  h<sup>-1</sup> to 8.24 at  $D = 0.08$  h<sup>-1</sup> and gave a linear graph (fitted by regression analysis) when plotted against  $D$ . This plot gave an alternative means of calculating  $m$  (the  $q$  intercept of the  $q$  v.  $D$  plot) as 1.17 and  $Y_0$  (the reciprocal of the slope of the  $q$  v.  $D$  plot) as 11.1. Mean values from the two procedures for determining  $Y_0$  and  $m$  were thus 11.37 g dry wt (mol thiosulphate)<sup>-1</sup> and 1.29 mmol thiosulphate h<sup>-1</sup> (g dry wt)<sup>-1</sup>.

A typical substrate and product balance for these steady states was given at  $D = 0.06$  h<sup>-1</sup> at which the oxidation of 17.07 mM-thiosulphate was accompanied by the disappearance of 22.75 mM-nitrate and the production of 155 mg dry wt bacteria, giving a yield of 9.08 g (mol thiosulphate)<sup>-1</sup>. This corresponds to a nitrate:thiosulphate ratio of 1.33, lower than the theoretical ratio of 1.6 given by the oxidation equation. The production of 155 mg biomass is, however, equivalent to 73.5 mg C (6 mmol CO<sub>2</sub>) fixed and consequently to the use

pH 7.0. For anaerobic growth on solution of NaNO<sub>2</sub> was separately bulk medium between nitrite and thiosulphate-free basal medium feed in at pH 3.5, the two mixing in the anaerobic thiosulphate-limitation,

in an LH modular type series 500 volume of 750 ml, provided with on and dissolved oxygen monitor-ter with a working volume of 3 l maintained at pH 7.0 by automatic with 5% (v/v) CO<sub>2</sub> in N<sub>2</sub> at 15 ml under N<sub>2</sub>. The effluent gas from the pyrogallol. Medium solutions were for aerobic and anaerobic cultures, on Marlow, Falmouth, Cornwall). by controlling the flow rate into culture vessels was minimized by

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nt of thiosulphate, trithionate and was determined by titration with (1957). Nitrite was determined by re mixed with 1 ml 0.3 M-CdSO<sub>4</sub>, 0% (v/v) acetic acid and of 0.1 g 0% acetic acid), and made up to some interference by thiosulphate with Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> to a total concentra-ate and nitrite containing 12 mM-olved in acetone and estimated

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re continually flushed with y using a medium containing was the limiting nutrient in osulphate respiration by *T.*

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of 3 mmol thiosulphate to provide reducing equivalents for fixation (see Discussion). Thus the thiosulphate consumed for energetic purposes (i.e. nitrate reduction) was only 17–3 i.e. 14 mmol, giving a true nitrate:thiosulphate ratio of 1.62, in accord with the equation of thiosulphate oxidation.

*Anaerobic nitrate-limited chemostat culture growing on tetrathionate instead of thiosulphate*

Replacing thiosulphate by 11.4 mM- $K_2S_4O_8$  under the conditions described in the preceding section resulted in easy transition to steady states in which more than 95% of the tetrathionate was consumed and yield values at  $D = 0.025$ , 0.05 and  $0.076 \text{ h}^{-1}$  were, respectively, 18.06, 19.56 and  $20.70 \text{ g dry wt (mol tetrathionate)}^{-1}$ , indicating a  $Y_{\text{O}}$  of  $21.5 \text{ g dry wt (mol tetrathionate)}^{-1}$ .

*Anaerobic nitrite-limited chemostat culture*

A nitrate-limited culture was switched to a supply of 21 mM-thiosulphate with 20 mM-nitrite and steady states were sustained at dilution rates of  $0.07$  and  $0.08 \text{ h}^{-1}$ . Washout occurred at about  $D = 0.08 \text{ h}^{-1}$ . Nitrite was completely consumed, but small amounts (0.87 mM) of trithionate were detected along with 7.51 mM unused thiosulphate. The steady state biomass at  $D = 0.07$  was  $149 \text{ mg dry wt l}^{-1}$ , indicating a yield of  $12.23 \text{ g (mol thiosulphate)}^{-1}$ .

*Anaerobic thiosulphate-limited chemostat culture*

With thiosulphate (10 to 20 mM) as the limiting nutrient and nitrate supplied in excess at 30 mM, virtually all the input thiosulphate was completely oxidized with no polythionate or sulphur formation (Tables 1 and 2). Nitrate consumption frequently exceeded the theoretical requirement, while nitrite formation and disappearance showed a harmonic oscillation at fixed dilution rates, with little dampening at higher  $D$  values. The effluent gases from the culture contained small amounts of  $N_2O$  and  $NO$  (detected as  $NO_x$ ). Eight steady states were maintained between  $D = 0.021$  and  $0.083 \text{ h}^{-1}$ . Washout occurred at  $D = 0.09 \text{ h}^{-1}$ . Biomass and yield tended to increase at higher  $D$  values in a similar manner to the nitrate-limited cultures (Table 1). The biomass values indicated growth efficiency similar to that of nitrate-limited cultures, as was previously shown at a single dilution rate ( $0.03 \text{ h}^{-1}$ ) by Timmer-ten-Hoor (1976). Greater variability in yield (and apparent protein content of cultures) was found with thiosulphate-limited anaerobic cultures than with those limited by nitrate, possibly due to variable nitrite accumulation. Consequently determination of  $Y_{\text{O}}$  was somewhat imprecise and values between  $9.2$  and  $14.2 \text{ g dry wt (mol thiosulphate)}^{-1}$  could be deduced from the data (mean  $11.7$ ). A plot of the  $q$  v.  $D$  data of Table 1 indicated  $Y_{\text{O}} = 9.22 \text{ g dry wt (mol thiosulphate)}^{-1}$  and  $m = 1.20 \text{ mmol thiosulphate h}^{-1} (\text{g dry wt})^{-1}$ , calculated by linear regression analysis.

*Aerobic thiosulphate-limited chemostat culture*

In two chemostat runs, eight steady states were established at  $D = 0.02$  to  $0.13 \text{ h}^{-1}$  with culture washout between  $0.13$  and  $0.14 \text{ h}^{-1}$ . Thiosulphate was completely consumed in all steady states and was the growth-limiting nutrient (Table 1). However, aeration with air alone [at  $250 \text{ ml (l culture volume)}^{-1} \text{ min}^{-1}$ ] was also  $CO_2$  limiting as the yield at  $D = 0.02 \text{ h}^{-1}$  was increased from  $7.5 \text{ g dry wt mol}^{-1}$  with air to  $11.8$  when the gas flow was supplemented with 5% (v/v)  $CO_2$ . A similar phenomenon was seen with *T. ferrooxidans* grown on tetrathionate (Eccleston & Kelly, 1978) and presumably indicates dependence on  $CO_2$  concentration of the efficiency of energy coupling during thiosulphate oxidation. All subsequent steady states were attained with excess  $CO_2$  supply and showed an increase in steady state yield with increasing dilution rate from  $10.7 \text{ g dry wt (mol thiosulphate)}^{-1}$  at  $D = 0.02$  to  $12.8$  at  $D = 0.08 \text{ h}^{-1}$ .  $Y_{\text{O}}$  was  $14.7 \text{ g dry wt (mol thiosulphate)}^{-1}$  and  $m$  was  $0.57 \text{ mmol thiosulphate h}^{-1} (\text{g dry wt})^{-1}$ , as calculated from a plot of  $q$  v.  $D$ .

Table 1  
denitrification  
thiosulphate

Dilution rate* ( $\text{h}^{-1}$ )
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Table 2

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Table 1. Comparison of steady state biomass and thiosulphate consumption by *Thiobacillus denitrificans* in (A) nitrate-limited anaerobic, (B) thiosulphate-limited anaerobic and (C) thiosulphate-limited aerobic cultures

Input thiosulphate concentrations were 20 mm for A and C, and 17 mm for B.

Dilution rate* (h <sup>-1</sup> )	Steady state biomass (mg dry wt l <sup>-1</sup> )			Steady state thiosulphate consumption (mmol l <sup>-1</sup> )			$q_{s,0.5}$ [mmol h <sup>-1</sup> (g dry wt) <sup>-1</sup> ]		
	A	B†	C†	A	B	C	A	B†	C†
0.02	115	119	211	16.4	15.4	19.7	2.85	2.82	1.88
0.03	125	117	ND	16.4	17.0	—	3.95	4.37	—
0.04	135	100	256	16.3	17.0	19.7	4.83	6.80	3.08
0.05	145	123	ND	16.0	17.0	—	5.52	6.92	—
0.06	155	142	200	17.1	17.0	19.5	6.61	7.18	5.85
0.07	160	147	ND	17.2	17.0	—	7.51	8.10	—
0.08	180	132	250	18.5	17.0	19.5	8.24	10.30	6.24
0.10	w	w	340	—	—	19.5	—	—	5.74

w, Culture washout; ND, not determined.

\* Approximate dilution rates given for ease of comparison: actual values employed were up to 5% greater than indicated.

† Mean values from more than one steady state determination.

Table 2. Effect of input nutrient concentration on steady state conditions and yield of anaerobic cultures of *Thiobacillus denitrificans* at constant dilution rate (0.08 h<sup>-1</sup>)

Between six and eight volume changes of each medium were passed through the culture to establish true steady states before changing to the next medium mixture. Growth yields are expressed as g dry wt (mol thiosulphate)<sup>-1</sup>.

Input concn (mm)			Steady state concn (mm)			Biomass (mg l <sup>-1</sup> )	Yield
Thiosulphate	Nitrate	Nitrite	Thiosulphate	Nitrate	Nitrite		
20.14	19.78	0	1.61	0.89	0.007	180	9.71
20.14	29.67	0	1.25	2.47	0	180	9.53
10.07	14.84	0	0.63	0.92	0	90	9.53
10.07	29.67	0	0	8.78	0.025	85	8.44
20.14	29.67	0	1.25	2.97	0	180	9.53
40.28	29.67	0	21.25	0.59	0.023	c. 45	c. 2.37
40.28	29.67	5.0	16.25	0.69	0.015	110	4.58

#### Specific rates of thiosulphate consumption

As described,  $q_{s,0.5}$  was used as a means of calculating  $Y_0$  and  $m$ . Values of  $q_{s,0.5}$  increased with  $D$  for all three conditions studied (Table 1). Over the range 0.02 to 0.08 h<sup>-1</sup> these values ranged from 1.9 to 10.3 mmol h<sup>-1</sup> (g dry wt)<sup>-1</sup> for the three growth conditions. Linear regression analysis fits of seven or eight values for each growth state indicated  $m$  values between 0.6 (aerobic) and 1.2 (anaerobic, nitrate- or thiosulphate-limited) mmol thiosulphate h<sup>-1</sup> (g dry wt)<sup>-1</sup>.

#### Responses in anaerobic chemostat cultures to variation of nutrient supplies

Alteration of the input concentration of thiosulphate or nitrate demonstrated that each could be made growth-limiting (Table 2). When a large excess of thiosulphate was supplied under nitrate limitation, culture lysis was observed with a low steady state biomass. Supplementation with a small amount of nitrite, allowing more thiosulphate metabolism, partially alleviated this effect (Table 2).

### Elemental composition of *Thiobacillus denitrificans*

Organisms from chemostats operating at five different dilution rates (between 0.02 and 0.08 h<sup>-1</sup>) grown anaerobically or aerobically with thiosulphate-limitation or anaerobically with nitrate-limitation all had essentially the same content of carbon, hydrogen and nitrogen as a percentage of the dry wt. From 14 separate analyses, the composition [% (w/w)  $\pm$  S.E.M.] was: carbon, 47.40  $\pm$  1.02; hydrogen, 6.88  $\pm$  0.23; nitrogen, 12.70  $\pm$  0.79. The C/N ratios for the three culture conditions (A, B and C) were 3.52, 3.87 and 3.63, respectively. We conclude that growth rate and condition did not significantly affect the gross composition of the organism.

### DISCUSSION

Our observations demonstrate that *Thiobacillus denitrificans* can grow efficiently in chemostat culture under several conditions of nutrient limitation both anaerobically and aerobically. Our data for growth yield and maintenance enable us to estimate the relative growth efficiencies and the amounts of ATP generated by anaerobic and aerobic thiosulphate oxidation. The true growth yield of 11.37 g dry wt (mol thiosulphate)<sup>-1</sup> for anaerobic nitrate-limited culture can be compared with the observed maximum yield (at  $D = 0.08$ , uncorrected for  $m$ ) of 9.7 and the average yields of 9.3 and 5.7 that can be calculated from the data for batch cultures reported by Taylor *et al.* (1971) and Lieske (1912). Timmer-ten-Hoor (1976), using a continuous culture at  $D = 0.03$  h<sup>-1</sup>, obtained 9.26 under similar conditions.

In anaerobic cultures, growth yields (in terms of g protein or dry wt per mol thiosulphate consumed) were comparable with either nitrate- or thiosulphate-limitation (as reported for a single fixed dilution rate by Timmer-ten-Hoor, 1976), whereas the yield was higher in aerobic thiosulphate-limited culture, giving  $Y_o$  values [g dry wt (mol thiosulphate)<sup>-1</sup>] of 11.37 for anaerobic (nitrate-limited) and 14.69 for aerobic cultures, as expected from the greater energy available from the aerobic oxidation of thiosulphate (Timmer-ten-Hoor, 1976; Kelly, 1978). These  $Y_o$  values are comparable with the higher of two values reported by Hemphling & Vishniac (1967), but are considerably higher than more recently determined values for aerobic *T. neapolitanus* of 5.27 (Kelly, unpublished) and 6.5 (J. G. Kuenen, personal communication); 7.48 for *T. ferrooxidans* (Kelly, Eccleston & Jones, 1977; Eccleston & Kelly, 1976, 1978); 5.2 for *Thiomicrospira pelophila* and about 7 for *Thiobacillus*  $\Delta 2$  (J. G. Kuenen, personal communication). The values for maintenance coefficient ( $m$ ) are comparable with others reported recently (Justin & Kelly, 1976) and from our subsequent calculations are equivalent to  $m_{ATP}$  values in the range 4 to 11 mmol h<sup>-1</sup> (g dry wt)<sup>-1</sup>. There was indication from some of our data that  $m$  might decrease at lower dilution rates.

Using  $Y_o$  values around 7 for *T. ferrooxidans* growing on thiosulphate, we calculated (Kelly *et al.*, 1977; Eccleston & Kelly, 1978) that aerobic thiosulphate oxidation supported synthesis of only 3 mol ATP per mol, and that, while 2 mol ATP could be produced by electron transport phosphorylation, it was possible that the oxidation of the sulphane-sulphur of thiosulphate was not energy-conserving. This would be consistent with the operation of a non-energy-linked oxygenase system in aerobic thiobacilli for the conversion of sulphur to sulphite (Suzuki & Silver, 1966; Silver & Lundgren, 1968; Taylor, 1968). The considerably higher values reliably reported now for *T. denitrificans* in this paper and previously (Justin & Kelly, 1976; Timmer-ten-Hoor, 1976) indicate that more efficient energy coupling systems may exist in this facultatively anaerobic thiobacillus.

At a carbon content of 47.4% (w/w) of the dry wt, the anaerobic  $Y_o$  of 11.37 indicates the fixation of 5.39 g carbon or 0.45 mol CO<sub>2</sub> per mol thiosulphate. By the Calvin cycle this requires 0.9 mol NADH and 1.35 mol ATP. Since the oxidation of 1 mol thiosulphate generates 8 reducing equivalents (H), and 0.9 NADH requires 1.8 (H) for its formation, only 6.2 (H) are available for energy coupling by electron transport phosphorylation. Conse-

quently, this is a conservative estimate of the biosynthetic capacity of the organism. The C/N ratio of 3.63 indicates that 4.57 mol terminal electron acceptor are required for the conversion of 1 mol thiosulphate to NADH. The C/N ratio of 3.63 indicates that 4.57 mol terminal electron acceptor are required for the conversion of 1 mol thiosulphate to NADH. The C/N ratio of 3.63 indicates that 4.57 mol terminal electron acceptor are required for the conversion of 1 mol thiosulphate to NADH.

These values are comparable with those reported for other aerobic thiobacilli (e.g. 14.66 g dry wt (mol thiosulphate)<sup>-1</sup> for *T. ferrooxidans*).

There is a close link between the growth yield and the maintenance coefficient, and that the maintenance coefficient is a function of the growth rate.

The values for maintenance coefficient ( $m$ ) are equivalent to those reported for other aerobic thiobacilli (e.g. 7.48 for *T. ferrooxidans*).

The values for maintenance coefficient ( $m$ ) are equivalent to those reported for other aerobic thiobacilli (e.g. 7.48 for *T. ferrooxidans*).

We thank Dr G. R. J. (1976) for his contribution to this work.

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quently, the observed  $Y_0$  and fixation of 0.45 mol  $\text{CO}_2$  per mol thiosulphate means that this is supported energetically by the oxidation of 0.775 mol thiosulphate. The fixation and conversion of 0.45 mol  $\text{CO}_2$  to the level of cell constituents also requires 0.39 mol ATP for biosynthesis from the hexose level (Stouthamer, 1973), and the reduction of 0.9 mol NAD probably requires 1.8 mol ATP to effect electron transport from the level of cytochrome *c* to NAD<sup>+</sup> (assuming 2 mol ATP required per mol NAD<sup>+</sup> reduced). Consequently, total ATP indicated to be available from the oxidation of 0.775 mol thiosulphate was 3.54 mol, i.e. 4.57 mol ATP per mol thiosulphate oxidized for energetic purposes with nitrate as the terminal electron acceptor. Aerobically, the  $Y_0$  of 14.69 can be calculated to indicate 6.43 mol ATP per mol thiosulphate oxidized. In both these calculations, if NAD<sup>+</sup> reduction requires only one mol ATP per mol, the ATP yields are reduced to 3.41 and 5.27 for anaerobic and aerobic growth, respectively. Taking the higher values and assuming that they represent 4 to 5 and 6 to 7 mol ATP formed per mol thiosulphate, respectively, and that 1 to 2 mol ATP are formed in each case by substrate level phosphorylation (Peck, 1968), at least 3 and 5 mol ATP are formed by oxidative phosphorylation anaerobically and aerobically, respectively. This must indicate that the oxidation of the sulphane-sulphur of thiosulphate supports phosphorylation (Kelly, 1978) and hence is not effected by the sulphur oxygenase: this could in any case not be significant during anaerobic growth.

These calculations indicate that for each mol thiosulphate used for energetic purposes [i.e. corrected for (H) requirement for  $\text{CO}_2$  fixation] the amount of growth supported is 14.66 g dry wt under anaerobic nitrate-limitation and 20.69 g dry wt under aerobic thiosulphate-limitation. Anaerobically, available energy would seem to be 71% of that available aerobically.

Thermodynamically, the theoretically available free energy for nitrate-linked and oxygen-linked thiosulphate oxidation is 741 and 936 kJ mol<sup>-1</sup>, respectively (Kelly, 1978), indicating that no better than 79.2% of the aerobic growth yield would be expected anaerobically, in moderate agreement with the observed result.

The anaerobic nitrate-limited  $Y_0$  values for thiosulphate (11.37) and tetrathionate (21.5) are equivalent to, respectively, 14.66 and 28.39 g dry wt per mol thiosulphate or tetrathionate oxidized for energetic purposes, indicating that the energy available from thiosulphate oxidation is 51.7% of that available from tetrathionate oxidation, in reasonable agreement with the relative free energy available from their oxidation of 54 to 56% (Kelly, 1978).

The detailed response of *T. denitrificans* to progressive transition from aerobic to anaerobic continuous culture is described in the following paper (Justin & Kelly, 1978).

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## Metabolic Changes in *Thiobacillus denitrificans* Accompanying the Transition from Aerobic to Anaerobic Growth in Continuous Chemostat Culture

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*Thiobacillus denitrificans* grew exclusively aerobically in thiosulphate-limited chemostat culture at all dissolved oxygen concentrations between 12 and 216  $\mu\text{M}$ . Nitrate reduction did not occur in aerobic cultures and nitrate and nitrite reductases only reached high levels under complete anaerobiosis. Growth yield was greatest [11.50 g dry wt (mol thiosulphate oxidized)<sup>-1</sup>] at the lowest dissolved oxygen concentration (12  $\mu\text{M}$ ) and decreased at higher dissolved oxygen concentrations, indicating oxygen to be a growth-inhibitory substrate; the anaerobic yield was only 77% of the maximum aerobic yield (all tested at a dilution rate of 0.08 h<sup>-1</sup>) in agreement with thermodynamic calculations. Low activities of thiosulphate-oxidizing enzyme and sulphur-oxidizing enzyme were detected in aerobic cultures, but activities were even lower in anaerobic cultures. Efficient energy coupling mechanisms with respect to sulphur and thiosulphate oxidations are indicated.

### INTRODUCTION

*Thiobacillus denitrificans* is the only well-established facultatively anaerobic thiobacillus and is capable of rapid growth on thiosulphate both aerobically and anaerobically with nitrate or nitrite as oxidant (Justin & Kelly, 1978). No study has been reported on the biochemical and physiological changes in the organism accompanying the transition from aerobic to anaerobic growth conditions. The claim has been made that prolonged aerobic culture results in the loss of capacity to grow anaerobically (Vishniac & Santer, 1957; Woolley, Jones & Happold, 1962), but this has never been reliably substantiated. Elsewhere (Eccleston & Kelly, 1978; Justin & Kelly, 1978), we have speculated that during aerobic growth of *T. denitrificans* more energy is conserved during thiosulphate oxidation than during growth of other aerobic thiobacilli. The large aerobic growth yield of *T. denitrificans* would be consistent with the conservation of energy during oxidation of the sulphane-sulphur of thiosulphate but not when this is oxidized, for example, by *T. ferrooxidans* (Eccleston & Kelly, 1978). This could occur if the sulphur-oxidizing oxygenase enzyme found in aerobic thiobacilli (Suzuki, 1965*a, b*; Suzuki & Silver, 1966; Charles & Suzuki, 1966; Silver & Lundgren, 1968*a*), which has been postulated to play a central role in sulphane-sulphur oxidation (Kelly, 1968; Roy & Trudinger, 1969), was not important in aerobic thiosulphate oxidation by *T. denitrificans*. This enzyme could not in any case have a role during anaerobic growth.

### METHODS

**Organism, continuous chemostat culture and analytical methods.** These were as described previously (Justin & Kelly, 1978). Dissolved oxygen in chemostat cultures was controlled by varying the composition of the gas phase flowing through the culture: 5% (v/v) CO<sub>2</sub> in air was used for fully aerobic cultures, and was progressively supplemented with 5% (v/v) CO<sub>2</sub> in N<sub>2</sub> so that anaerobic cultures received only the CO<sub>2</sub>/N<sub>2</sub>

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mixture. Dissolved oxygen was measured with a Clark oxygen electrode and LH Engineering (Slough, Buckinghamshire) dissolved oxygen module, reading dissolved oxygen as a percentage of air saturation. These values were converted to  $\mu\text{M-O}_2$  assuming that air-saturated medium contained  $245 \mu\text{M-O}_2$  (Tuovinen & Kelly, 1972). Each new steady state was established by passing six to eight culture volumes of medium through the vessel at a dilution rate of  $0.08 \text{ h}^{-1}$ . The input medium usually contained  $18.69 \text{ mM-Na}_2\text{S}_2\text{O}_4$  and  $24 \text{ mM-NaNO}_3$  for aerobic thiosulphate- and anaerobic nitrate-limitation, but in some cases  $40 \text{ mM-NaNO}_3$  or  $10 \text{ mM-K}_2\text{S}_2\text{O}_8$  was used instead of nitrate or thiosulphate, respectively.

**Preparation of crude extract for enzyme assays.** Organisms were harvested from steady state chemostats by centrifuging at  $4^\circ\text{C}$ , washed with water, recentrifuged and the pellet was stored at  $-20^\circ\text{C}$  or used immediately for enzyme assay. Organisms were suspended ( $25\%$ , v/v) in  $0.05 \text{ M-Tris-HCl}$  buffer, pH 7.54, and passed twice through an Aminco French pressure cell at  $140 \text{ kPa}$ . The broken suspension was centrifuged at  $10000 \text{ g}$  for  $30 \text{ min}$  at  $4^\circ\text{C}$  and the supernatant liquid was used for enzyme assays.

**Nitrite reductase.** Nitrite production from nitrate was measured using NADH as electron donor (Sawhney & Nicholas, 1977) in reaction mixtures in test tubes containing (final vol.  $2 \text{ ml}$ ):  $\text{Tris-HCl}$ , pH 7.54,  $80 \mu\text{mol}$ ; phenazine methosulphate,  $1 \mu\text{mol}$ ;  $\text{KNO}_3$ ,  $1 \mu\text{mol}$ ; crude extract,  $0.01 \text{ ml}$ . After equilibration at  $30^\circ\text{C}$  for  $2 \text{ min}$ , the reaction was initiated by adding  $1 \mu\text{mol}$  NADH, and terminated after  $15 \text{ min}$  by adding  $0.1 \text{ ml}$   $1 \text{ M-acetaldehyde}$  and  $0.03 \text{ mg}$  alcohol dehydrogenase in  $0.1 \text{ ml}$   $0.05 \text{ M-Tris-HCl}$ , pH 7.54, to oxidize the residual NADH. Nitrite was determined by the Griess-Ilosvay method (Justin & Kelly, 1978). Activity was expressed as  $\text{nmol NO}_2^- \text{ produced min}^{-1} (\text{mg protein})^{-1}$ .

**Nitrite reductase.** Activity was measured at  $30^\circ\text{C}$  in  $10 \text{ ml}$  Warburg flasks (Aminuddin & Nicholas, 1973) containing (final vol.  $1 \text{ ml}$ ):  $\text{Tris-HCl}$ , pH 7.54,  $40 \mu\text{mol}$ ; phenazine methosulphate,  $1 \mu\text{mol}$ ;  $\text{NaNO}_3$ ,  $0.6 \mu\text{mol}$ ; crude extract,  $0.01 \text{ ml}$ . The reaction was initiated by adding  $1 \mu\text{mol}$  NADH, after flushing the flasks with  $\text{O}_2$ -free  $\text{N}_2$  for  $10 \text{ min}$ . The reaction was terminated after  $0, 3, 6$  or  $10 \text{ min}$  as for nitrate reductase assays and nitrite was estimated. Activity was expressed as  $\text{nmol NO}_2^- \text{ reduced min}^{-1} (\text{mg protein})^{-1}$ .

**Thiosulphate-oxidizing enzyme.** Ferricyanide reduction (Trudinger, 1961) was measured spectrophotometrically with a reaction mixture in  $1 \text{ cm}$  cuvettes containing (final vol.  $3 \text{ ml}$ ): potassium phthalate buffer, pH 5.0,  $300 \mu\text{mol}$ ;  $\text{Na}_2\text{S}_2\text{O}_4$ ,  $30 \mu\text{mol}$ ; potassium ferricyanide,  $3 \mu\text{mol}$ ; crude extract,  $0.05 \text{ ml}$ . Thiosulphate and ferricyanide were omitted from the reference cuvette. The reaction was initiated by adding the extract. Activity was measured for up to  $60 \text{ min}$  and expressed as  $\mu\text{mol ferricyanide reduced min}^{-1} (\text{mg protein})^{-1}$ .

**Sulphur-oxidizing enzyme.** This was assayed by measuring thiosulphate production and oxygen uptake using Warburg manometers (Suzuki & Silver, 1966) and reaction mixtures containing (final vol.  $2 \text{ ml}$ ):  $\text{Tris-HCl}$ , pH 7.8,  $500 \mu\text{mol}$ ; sulphur (BDH 'Optran' grade),  $48 \text{ mg}$ ; 2,2'-bipyridyl,  $0.2 \mu\text{mol}$ ; catalase,  $250 \mu\text{g}$ ; crude extract,  $0.1 \text{ ml}$ . The reaction in air-filled flasks was initiated by adding  $5 \mu\text{mol}$  reduced glutathione. After  $210 \text{ min}$ , during which oxygen uptake was recorded, flask contents were sampled and thiosulphate was determined cyanolytically as ferric thiocyanate (Suzuki, 1965a).

**Anaerobic nitrite reduction by suspensions of *Thiobacillus denitrificans*.** From steady state cultures held at different dissolved oxygen concentrations,  $200 \text{ ml}$  samples were removed and the organisms were harvested by centrifuging, washed and resuspended in salts solution lacking thiosulphate and nitrate. Suspensions in Universal bottles sealed with Suba-seal vaccine stoppers were made anaerobic by passage of  $\text{N}_2$  and incubated at  $30^\circ\text{C}$ .  $\text{Na}_2\text{S}_2\text{O}_4$  ( $2 \mu\text{mol ml}^{-1}$ ) and  $\text{NaNO}_3$  ( $1.8 \mu\text{mol ml}^{-1}$ ) were added and samples were removed for thiosulphate and nitrite analysis at intervals for  $300 \text{ min}$ .

## RESULTS

### Effect of dissolved oxygen concentration on biomass, substrate consumption and yield in steady state chemostats

Using chemostat cultures at a fixed dilution rate of  $0.08 \text{ h}^{-1}$  with a medium containing about  $19 \text{ mM-thiosulphate}$  and about  $24 \text{ mM-nitrate}$ , a series of steady states was established at different dissolved oxygen concentrations. The consequence of progressively decreasing the steady state dissolved oxygen from  $88\%$  of air saturation ( $216 \mu\text{M-O}_2$ ) in a fully aerobic culture to  $5\%$  of air saturation ( $12 \mu\text{M-O}_2$ ) was to increase the steady state biomass without decreasing consumption of the limiting nutrient, thiosulphate, which was completely consumed at all dissolved oxygen concentrations. Consequently the yield increased with decreasing steady state dissolved oxygen, while the specific rate of thiosulphate oxidation ( $q_{\text{thio}}$ ) decreased (Table 1). At all dissolved oxygen concentrations tested, there was no significant reduction of nitrate, although trace amounts of nitrite were possibly formed at  $12$  to  $22 \mu\text{M}$  (Table 1). The change from  $12 \mu\text{M-O}_2$  to nitrate-limited anaerobiosis resulted in

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Table 1. Effect of dissolved oxygen concentration on biomass, substrate consumption and yield in steady state chemostat cultures of *Thiobacillus denitrificans* at  $D = 0.08 \text{ h}^{-1}$ 

Growth yields are expressed as g dry wt (mol thiosulphate oxidized) $^{-1}$  and specific rates of thiosulphate oxidation ( $q_{\text{thio}}$ ) as mmol (g dry wt) $^{-1} \text{ h}^{-1}$ .

Dissolved oxygen concn ( $\mu\text{M}$ )	Biomass (mg dry wt $\text{l}^{-1}$ )	Thiosulphate consumed (mmol $\text{l}^{-1}$ )	Growth yield	$q_{\text{thio}}$	Nitrate consumed (mmol $\text{l}^{-1}$ )	Nitrite produced (mmol $\text{l}^{-1}$ )
216	155	18.69	8.29	9.65	0	0.015
191	145	18.69	7.76	10.31	0	0.020
167	165	18.69	8.83	9.06	0	0.013
135	175	18.69	9.36	8.54	0	0.012
108	182	18.69	9.74	8.22	0	0.011
74	175	18.69	9.36	8.54	1.1	0.021
54	200	18.69	10.70	7.48	0	0.030
22	205	18.69	10.97	7.29	0	0.117
12	215	18.69	11.50	6.95	0	0.156
0	150	16.06	9.33	8.56	23.4	0.171

immediate nitrate reduction and transitory accumulation of sulphur and nitrite within 4 h. When the steady state was established, sulphur was absent and nitrite negligible, although, as expected, about 2.6 mM-thiosulphate remained unconsumed (Table 1). Re-aerating the anaerobic culture to establish 80% air saturation concentrations of  $\text{O}_2$  resulted in complete consumption of thiosulphate and suppression of nitrate reduction.

#### Nitrite reduction by suspensions of organisms grown with different concentrations of dissolved oxygen

Organisms harvested from steady states held at 11 different dissolved oxygen concentrations were tested for their ability to reduce nitrate or nitrite anaerobically in the presence of thiosulphate. Only organisms grown fully anaerobically in nitrate-limited culture showed any nitrate reduction activity. Organisms from cultures held at 68% air saturation (167  $\mu\text{M-O}_2$ ) and above did not reduce nitrite anaerobically during 5 h incubation at 30 °C. Organisms grown at lower dissolved oxygen concentrations reduced nitrite after a lag, the length of which was a function of the dissolved oxygen regime in the culture from which the organisms were harvested (Fig. 1). There was no lag with organisms grown anaerobically with nitrate-limitation.

#### Nitrate and nitrite reductase activities

These were assayed in crude extracts using NADH as reductant. Activities of both enzymes were negligible in cultures maintained at dissolved oxygen concentrations equivalent to 52% air saturation (127  $\mu\text{M-O}_2$ ) and above (Table 2). Nitrate reductase increased slightly below 54  $\mu\text{M-O}_2$  and to 10 times the aerobic level under complete anaerobiosis. Nitrite reductase activity increased considerably as the dissolved oxygen concentration was lowered from 91 to 14  $\mu\text{M-O}_2$ , but rose to very high levels in anaerobic cultures (Table 2). Using freshly harvested anaerobic organisms assayed soon after breakage in the French pressure cell, the maximum nitrate reductase activity was 28.6 nmol  $\text{min}^{-1}$  (mg protein) $^{-1}$  and nitrite reductase activity was 264.0 nmol  $\text{min}^{-1}$  (mg protein) $^{-1}$ .

Both enzymes were reasonably stable when stored at -20 °C. Nitrate reductase was not raised above its aerobic levels in anaerobic organisms cultured in nitrate-free nitrite-limited medium, but nitrite reductase reached a level comparable to that under nitrate-limited anaerobiosis (Table 2). Substituting tetrathionate for the usual thiosulphate substrate (Justin & Kelly, 1978) under anaerobic nitrate-limitation resulted in enzyme activities comparable to those in thiosulphate cultures (Table 2).

de and LH Engineering (Slough, as a percentage of air saturation. m contained 245  $\mu\text{M-O}_2$  (Tuovinen eight culture volumes of medium ally contained 18.69 mM- $\text{Na}_2\text{S}_2\text{O}_4$  itation, but in some cases 40 mM- ectively.

ated from steady state chemostats ut was stored at -20 °C or used 0.05 M-Tris HCl buffer, pH 7.54, broken suspension was centrifuged zyme assays.

NADH as electron donor (Sawhney ml): Tris HCl, pH 7.54, 80  $\mu\text{mol}$ ; ml. After equilibration at 30 °C for ted after 15 min by adding 0.1 ml Tris HCl, pH 7.54, to oxidize the Justin & Kelly, 1978). Activity was

ks (Aminuddin & Nicholas, 1973) methosulphate, 1  $\mu\text{mol}$ ;  $\text{NaNO}_3$ , 1  $\mu\text{mol}$  NADH, after flushing the 5 or 10 min as for nitrate reductase reduced  $\text{min}^{-1}$  (mg protein) $^{-1}$ . 161) was measured spectrophoto- 0 ml): potassium phthalate buffer, ude extract, 0.05 ml. Thiosulphate as initiated by adding the extract. ide reduced  $\text{min}^{-1}$  (mg protein) $^{-1}$ . te production and oxygen uptake ures containing (final vol. 2 ml): 2,2-bipyridyl, 0.2  $\mu\text{mol}$ ; catalase, 1 by adding 5  $\mu\text{mol}$  reduced gluta- contents were sampled and thio- 50).

From steady state cultures held at and the organisms were harvested phosphate and nitrate. Suspensions in erobic by passage of  $\text{N}_2$  and incu- added and samples were removed

#### Substrate consumption

with a medium containing steady states was established of progressively decreasing 216  $\mu\text{M-O}_2$  in a fully aerobic steady state biomass without ate, which was completely ally the yield increased with ate of thiosulphate oxidation ations tested, there was no ite were possibly formed at ited anaerobiosis resulted in

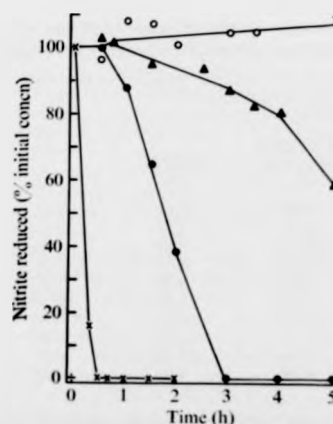


Fig. 1. Nitrite reduction by anaerobic suspensions of *Thiobacillus denitrificans* taken from chemostat cultures grown with different concentrations of dissolved oxygen. Consumption of nitrite, in the presence of 2 mM- $\text{Na}_2\text{S}_2\text{O}_4$ , is expressed as a percentage of the initial nitrite concentration (1.8 mM- $\text{NaNO}_2$ ). Steady state oxygen concentrations in the cultures from which the bacteria were taken were 216  $\mu\text{M}$  (○), 135  $\mu\text{M}$  (Δ), 74  $\mu\text{M}$  (●) and 0 (×).

Table 2. Effect of dissolved oxygen concentration on nitrate and nitrite reductase activities in crude extracts of *Thiobacillus denitrificans* from chemostat cultures at  $D = 0.08 \text{ h}^{-1}$

Dissolved oxygen concn ( $\mu\text{M}$ )	Growth-limiting substrate	Enzyme activity ( $\text{nmol min}^{-1} (\text{mg protein})^{-1}$ )	
		Nitrate reductase	Nitrite reductase
211	Thiosulphate	1.6	1.2
176	Thiosulphate	1.3	1.3
147	Thiosulphate	1.3	0
127	Thiosulphate	1.7	0
91	Thiosulphate	1.2	29.4
86	Thiosulphate	1.6	61.7
54	Thiosulphate	2.2	48.5
15	Thiosulphate	4.0	85.9
14	Thiosulphate	9.0	47.4
0	Nitrate	18.9	269.0
0	Nitrite	2.0	280.0
0	(nitrate-free medium)		
0	Nitrate ( $\text{K}_2\text{S}_2\text{O}_8$ instead of $\text{Na}_2\text{S}_2\text{O}_4$ )	15.8	241.0

#### Sulphur-oxidizing enzyme

Activities of this enzyme were very low although it was detected in both anaerobic and aerobic organisms (Table 3). In most cases the molar ratio of  $\text{O}_2$  consumed to thiosulphate formed was about 1.0, as required by the mechanism proposed by Suzuki (1965*a, b*) and Suzuki & Silver (1966). Freshly prepared crude extracts from freshly harvested aerobic (80% air saturation, 196  $\mu\text{M-O}_2$ ) and anaerobic cultures gave activities of 1.069 and 0.228  $\mu\text{mol thiosulphate formed (210 min)}^{-1} (\text{mg protein})^{-1}$ , respectively. The activity in crude extracts declined as the dissolved oxygen content of the cultures was lowered (Table 3). Mean values for six aerobic cultures (127 to 211  $\mu\text{M-O}_2$ ) and four anaerobic cultures were

Table 3. Crude extracts

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Table 3. Effect of dissolved oxygen concentration on sulphur-oxidizing enzyme activity in crude extracts of *Thiobacillus denitrificans* from chemostat cultures at  $D = 0.08 \text{ h}^{-1}$ 

Dissolved oxygen concn ( $\mu\text{M}$ )	Growth-limiting substrate	Enzyme activity [ $\mu\text{mol (210 min)}^{-1} (\text{mg protein})^{-1}$ ]	
		Oxygen uptake	Thiosulphate formation
211	Thiosulphate	0.42	0.44
176	Thiosulphate	0.61	0.63
147	Thiosulphate	0.50	0.31
127	Thiosulphate	0.39	0.92
91	Thiosulphate	0.47	0.45
86	Thiosulphate	0.51	0.24
54	Thiosulphate	0.28	0.11
22	Thiosulphate	0.35	0.40
15	Thiosulphate	0.31	0.36
14	Thiosulphate	0.14	0.14
0	Nitrate	0.18	0.17
0	Nitrite	0.41	0.16
0	(nitrate-free medium)		
0	Nitrate ( $\text{K}_2\text{S}_2\text{O}_8$ instead of $\text{Na}_2\text{S}_2\text{O}_8$ )	0.76	0.25

$0.740 \pm \text{s.e.m. } 0.32$  and  $0.202 \pm 0.038 \mu\text{mol (210 min)}^{-1} (\text{mg protein})^{-1}$ . Activity was dependent on the presence of both cell-free extract and reduced glutathione. In one test with an aerobic extract,  $1.20 \mu\text{mol}$  thiosulphate was formed by the complete assay mixture and  $0.27 \mu\text{mol}$  in the absence of glutathione, indicating some glutathione-independent activity, perhaps comparable with that studied by Taylor (1968) in *T. neapolitanus*.

#### Thiosulphate-oxidizing enzyme

This was detected in all steady state cultures, but in two freshly harvested and assayed aerobic cultures ( $196 \mu\text{M-O}_2$ ) activities were  $0.144$  and  $0.112 \mu\text{mol ferricyanide reduced min}^{-1} (\text{mg protein})^{-1}$ , whereas activity in a comparable anaerobic culture was only  $0.018$ . Activity was typically  $0.012$  to  $0.02$  in all anaerobic cultures. Activity was gradually lost on storage at  $-20^\circ\text{C}$ . These low activities should be compared with typical crude extract activities [ $\mu\text{mol ferricyanide reduced min}^{-1} (\text{mg protein})^{-1}$ ] of  $4.97$  for *T. neapolitanus* (Kelly, 1966),  $9.00$  for *T. ferrooxidans* (Silver & Lundgren, 1968b) and  $0.96$  for *T. thioparus* (Lyric & Suzuki, 1970).

#### DISCUSSION

The mechanism of sulphur-compound-dependent nitrate reduction in *T. denitrificans* is basically similar to that in other anaerobic nitrate-reducing bacteria (Adams, Warnes & Nicholas, 1971; Ishaque & Aleem, 1973; Baldensperger & Garcia, 1975; Sawhney & Nicholas, 1977). However, the regulation of the system under aerobic and anaerobic conditions has not previously been reported. Our experiments demonstrate that the levels of nitrate and nitrite reductase are regulated in response to oxygen tension, significant activities of nitrate reductase being detected only in microaerophilically and anaerobically cultured bacteria. Nitrite reductase was detected in cultures grown with  $91 \mu\text{M-O}_2$  but not with  $127 \mu\text{M-O}_2$ . For both enzymes, maximum activity was only found under complete anaerobiosis. Although nitrate and nitrite reductases were found in cultures held under low oxygen steady states, no nitrate reduction was detected in the cultures, indicating that oxygen is also inhibitory to nitrate reduction. Nitrate reductase synthesis also required the presence of nitrate, since cultures grown anaerobically with nitrite did not form significant nitrate reductase. These findings contrast with the metabolically similar *Thiomicrospira denitrificans*, in which nitrate reductase is constitutive (Timmer-ten-Hoor, 1977). The similar levels of nitrite

*denitrificans* taken from chemostat cultures. Consumption of nitrite, in the absence of initial nitrite concentration, was from which the bacteria were

and nitrite reductase activities in chemostat cultures at  $D = 0.08 \text{ h}^{-1}$

Enzyme activity [ $\mu\text{mol (210 min)}^{-1} (\text{mg protein})^{-1}$ ]

Nitrite reductase
1.2
1.3
0
0
29.4
61.7
48.5
85.9
47.4
269.0
280.0
241.0

detected in both anaerobic and aerobic cultures.  $\text{O}_2$  consumed to thiosulphate was by Suzuki (1965a, b) and in freshly harvested aerobic cultures activities of  $1.069$  and  $0.228$  respectively. The activity in crude extracts was lowered (Table 3). In four anaerobic cultures were

reductase found in anaerobic nitrate- or nitrite-limited chemostats suggested that this enzyme was fully derepressed under all the growth conditions tested.

The two enzymes possibly concerned in sulphur compound oxidation, the thiosulphate- and sulphur-oxidizing enzymes, were present at relatively very low levels in aerobic *T. denitrificans* and were repressed by 70 to 90% during anaerobic growth. The activity of thiosulphate-oxidizing enzyme in anaerobic cultures was only 0.3% of that in some aerobic thiobacilli (Kelly, 1966; Silver & Lundgren, 1968*b*) but similar to that in aerobic *Thiobacillus* A2 (Kelly, 1973). Earlier, Trudinger (personal communication) was unable to detect this enzyme in a different strain of *T. denitrificans*. The activity of the glutathione-dependent sulphur-oxidizing enzyme was maximally around 1  $\mu$ mol sulphur oxidized per mg protein in 210 min. This is of the same order as found for *T. thiooxidans*, *T. thioparus* and *T. ferrooxidans* (Suzuki, 1965*a, b*; Suzuki & Silver, 1966; Silver & Lundgren, 1968*a*) but is a very low activity. The organisms growing aerobically at a dilution rate of 0.08 h<sup>-1</sup> oxidized thiosulphate at a rate of 10.3 mmol (g dry wt)<sup>-1</sup> h<sup>-1</sup>. The protein content of aerobic bacteria was approximately 50% (w/w) of the dry wt, so the  $q_{s_{th}}$  was equivalent to 20.6  $\mu$ mol (mg total protein)<sup>-1</sup> h<sup>-1</sup>, or a rate some 80 times more rapid than could be accounted for by sulphur-oxidizing enzyme oxidation of the sulphane-sulphur of thiosulphate. We must conclude that our results do not support the view that the sulphur-oxidizing enzyme can be involved in thiosulphate metabolism in *T. denitrificans*. There is, moreover, no obvious role for the enzyme activity observed in anaerobic bacteria, unless this enzyme has another, as yet undetected, function.

*Thiobacillus denitrificans* can be switched back and forth in the chemostat between aerobic and anaerobic growth with no permanent loss of nitrate-reducing capacity during aerobiosis. It appears, however, that oxygen is an inhibitory substrate as aerobic growth yields increased progressively as the dissolved oxygen concentration was lowered. At the lowest oxygen level (12  $\mu$ M), the yield was 60 to 70% greater than that with 200  $\mu$ M-O<sub>2</sub>. The yield at 12  $\mu$ M-O<sub>2</sub> of 11.50 g dry wt per mol thiosulphate oxidized should be compared with the anaerobic yield, at the same dilution rate, of 9.33. Calculated as yield per mol thiosulphate oxidized for energetic purposes (Justin & Kelly, 1978), these values become 14.88 and 11.44 respectively, i.e. the anaerobic yield is 76.9% of the aerobic yield. This may be compared with 71% for relative  $Y_a$  values and 79.2% for theoretically available energy (Justin & Kelly, 1978), and probably indicates that dissolved oxygen at 12  $\mu$ M is optimal for the efficient aerobic growth of *T. denitrificans*. While *T. denitrificans* grows best at low dissolved oxygen concentrations it is still capable of producing greater growth yields at high dissolved oxygen than obligately aerobic thiobacilli (Justin & Kelly, 1978), probably by virtue of a more efficient energy-conserving sulphur oxidation mechanism.

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